The Role of *Clostridium botulinum* type C in the Pathogenesis of Equine Grass Sickness

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Abbreviations

Abbreviations used in this thesis

ADP = adenosine diphosphate
AGS = acute grass sickness
BCIP/NBT = 5-Bromo-4-chloro-3-9-indolyl phosphate and Nitro Blue Tetrazolium
BoNT = botulinum neurotoxin
BoNT/C = botulinum neurotoxin type C
BSA = bovine serum albumin
BVS = Bristol Veterinary School
C2I = C2 component I
C2II = C2 component II
CAMR = Centre for Applied Microbiological Research
CGS = chronic grass sickness
CHO = Chinese hamster ovary
CNS = central nervous system
CROP = clostridial repetitive oligopeptides
DNA = deoxyribose nucleic acid
dNTP = deoxynucleotide triphosphate
EDTA = ethylene diamine tetra-acetic acid
EGS = equine grass sickness
ELAW = equine leucocyte antigen workshop
ELISA = enzyme-linked immunosorbent assay
ELISPOT = enzyme-linked immunospot
EYAg = fastidious anaerobe agar containing 5% egg yolk emulsion and 10μg/ml gentamicin
FBS = foetal bovine serum
FITC = fluorescein isothiocyanate
GALT = gut associated lymphoid tissue
GAP = GTPase activating protein
GDF = GDI dissociation factor
GDI = GDP dissociation inhibitor
GDP = guanidine diphosphate
GEF = Guanidine nucleotide exchange factor

GHCl = guanidine hydrochloride

GI = gastrointestinal

GLC = gas liquid chromatography

GlcNAc = N-acetylglucosamine

GTP = guanidine triphosphate

H+L = heavy and light chains

HBSS = Hank's balanced salt solution

HRP = horseradish peroxidase

IEL = intraepithelial lymphocyte

ISL = Immune Systems Ltd.

LCT = large clostridial cytotoxin

LP = lamina propria

MPRL = Microbial Pathogenicity Research Laboratory

MW = molecular weight

NAD = nicotinamide adenine dinucleotide

NCTC = National Collection of Type Cultures

NGS = normal goat serum

NSF = N-ethylmaleimide-sensitive protein

NTNH = non-toxic non-haemagglutin

OD = optical density

PAGE = polyacrylamide gel electrophoresis

PBMC = peripheral blood mononuclear cells

PBS = phosphate buffered saline

PBSG = PBS containing 0.2% gelatin

PBS-T = PBS with 0.1% Tween 20

PBS-TF = PBS-T with 5% FBS

PCR = polymerase chain reaction

PMSF = phenylmethylsulphonyl fluoride

PP = Peyer's patches

PPY-G = protease peptone yeast extract broth containing 0.5% glucose

RER = rough endoplasmic reticulum

RID = radial immunodiffusion

RME = rabbit mucoid enteropathy

RNA = ribonucleic acid

rRNA = ribosomal ribonucleic acid

SC = secreting cells

SD = standard deviation

SDS = sodium dodecyl sulphate

SGS = subacute grass sickness

SNAP = soluble NSF fusion protein

TBS = tris buffered saline

TBS-T = TBS containing 0.1% Tween 20

TTBS = TBS containing 0.025% Tween 20

UDP = uracil diphosphate

VAMP = vesicle associated membrane protein

VFA = volatile fatty acid

Abstract

Equine grass sickness (EGS) is a fatal dysautonomia of unknown aetiology. It is characterised by severe neuronal degeneration and widespread neuronal loss in both the enteric and autonomic nervous systems. Similar dysautonomias have also been described in other animal species such as the cat and hare. This thesis investigates the hypothesis that EGS is caused by a toxicoinfection with *C. botulinum* type C, where the organism grows and produces toxin in the gastrointestinal tract (GI). The study has taken three main directions: (1) detection of the botulinum type C neurotoxin (BoNT/C) in the GI tract of horses with and without EGS, (2) isolation and characterisation of organisms phenotypically resembling *C. botulinum* type C, and (3) detection of antibodies to the type C neurotoxin and surface antigens in the serum, GI tract, colostrum and milk.

BoNT/C was detected by ELISA both directly in GI contents and after enrichment of the sample in order to determine the presence of both preformed toxin, and toxin-producing organisms. BoNT/C was detected by direct detection and/or enrichment in 74% of horses with acute grass sickness, 67% of horses with subacute grass sickness and 67% of horses with chronic grass sickness, compared to 10% of controls. BoNT/C was also detected directly and/or after enrichment in the GI tract of 81% cats with feline dysautonomia. These results support the hypothesis that toxicoinfection with *C. botulinum* type C is involved in the aetiology of EGS and possibly other similar dysautonomias, such as feline dysautonomia.

C. botulinum type C is phenotypically indistinguishable from *C. botulinum* type D and *Clostridium novyi* type A. These three organisms are grouped together as "Group III" botulinum strains and are differentiated on the basis of the major toxins they produce. However, the type C and D neurotoxins and the *C. novyi* alpha toxin are encoded on separate pseudolysogenic bacteriophages that can be readily lost. Loss of these phages results in loss of toxigenicity and non-toxigenic isolates are essentially indistinguishable from each other. Sixteen isolates phenotypically resembling Group III botulinum were isolated from the GI tract of nine horses, one cat and one hare. The animals from which these organisms were isolated all had some association with dysautonomia: five horses, one cat and one hare had dysautonomia at the time of sampling; two horses had recovered from EGS; two

horses had been in recent contact with EGS. None of the 16 isolates produced BoNT/C; the novyi alpha toxin gene was detected by PCR in three of the isolates. A novel method of distinguishing between *C. botulinum* type C/D and *C. novyi* type A was developed using antiserum raised against the surface antigens of *C. novyi* type A. This antiserum bound to a major immunogenic band that was present at 46kDa in *C. novyi* but absent in *C. botulinum* type C/D; a band at 43kDa was recognised in *C. botulinum* type C/D. By this method all 16 of the isolates were identified as *C. novyi* type A. There were striking similarities between the EDTA-extracted surface protein profiles of the isolates irrespective of species differences and the geographical distances between the animals from which the organisms were isolated. These isolates may have produced BoNT/C in vivo.

The immune response to the surface antigens of either *C. novyi* type A (serologically cross-reactive with *C. botulinum* type C/D) or *C. botulinum* type C and to BoNT/C was investigated at both the systemic and mucosal level. Horses with EGS had significantly lower levels of IgG both to surface antigens and BoNT/C; horses in contact with EGS had significantly higher levels of IgG to these antigens compared to horses with the disease. IgG to the surface antigens and BoNT/C was detected in the colostrum of all 36 mares investigated. These findings are in agreement with epidemiological findings that there is a reduced incidence of EGS in horses that have previously been in contact with the disease, and that suckling foals rarely get the disease. However, horses with EGS had significantly higher levels of IgA to BoNT/C in GI contents compared to controls, suggesting that horses with EGS had recently been exposed to the toxin in the GI tract.

Detection of BoNT/C, BoNT/C-producing organisms and IgA to BoNT/C in the GI tract of horses with EGS, support the hypothesis that toxicoinfection with *C. botulinum* type C is involved in the aetiology of EGS. There is also evidence that an antibody response to BoNT/C and surface antigens may protect horses in contact with EGS from developing the disease.

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Declaration

All of the experiments and procedures presented in this thesis were performed by the author unless otherwise indicated in the text.

Chapter One

Introduction

1.1 Equine Grass Sickness (EGS)

Equine grass sickness is a dysautonomia of unknown aetiology. It is usually fatal and at present is not preventable. The disease was first recognised in 1909 in army horses at Barry Camp, Forfarshire, Scotland. The majority of cases occur in Great Britain, with a higher incidence of the disease in Scotland (Milne et al, 1994); the disease is said to affect an estimated 2-4% of horses per annum (Cottrell et al, 1999). Grass sickness also occurs in Scandinavia and sporadically in other parts of northern Europe (Obel, 1955; Gilmour, 1987); cases have also been documented in Australia (Stewart, 1977) and the Falkland Islands (Woods and Gilmour, 1991). Mal Seco (dry sickness), an equine dysautonomia that occurs in Argentina and Chile, is thought to be identical to equine grass sickness (Uzal and Robles, 1993). In 1999, the first case of equine dysautonomia to occur in USA was confirmed (C. Hahn, personal communication).

The clinical, pathological and epidemiological features of the disease are now well recognised. The disease is characterised by severe neuronal degeneration and widespread neuronal loss in the enteric and autonomic nervous systems. However, despite almost a century's research into the aetiology of equine grass sickness, the cause of this disease is still not known.

1.1.1 Clinical signs

Equine grass sickness can occur in three forms - acute, subacute and chronic - classified on the basis of severity of clinical signs and duration of the disease (Doxey et al, 1991a). The mortality rate from grass sickness approaches approximately 90% (Pogson et al, 1992) – horses with acute and subacute grass sickness invariably die or are euthanased, whereas some horses with milder forms of chronic grass sickness can recover. Acute grass sickness is the most severe form of the disease with a sudden onset of clinical signs and death or euthanasia within 48 hours; horses with subacute grass sickness can survive up to seven days. Chronic grass sickness has a more insidious onset and can last from seven days up to several weeks or months, ending in either euthanasia or recovery.

The clinical symptoms of grass sickness have been extensively described (Greig, 1928; Edwards, 1987; Gilmour, 1988; Pinsent, 1989; Doxey et al, 1991a; Milne, 1997). The characteristic symptoms of the three categories of equine grass sickness are summarised in Table 1.1. The disease is characterised by dysfunction of the gastrointestinal (GI) tract. The severity and significance of this GI dysfunction is thought to account for the high mortality rate associated with the disease (Scholes et al, 1993a). There is a spectrum of symptoms within the three forms of grass sickness, with the clinical signs reflecting the extent of the neuronal damage. Horses therefore present with varying degrees of GI stasis/dysfunction, dysphagia (inability to swallow) and colic. In the acute form of the disease there can be complete GI stasis with impaction of the large colon and accumulation of fluid in the stomach and small intestine. Gastric reflux, either spontaneously or induced, of several litres of foul smelling fluid, is a characteristic of acute grass sickness.

Table 1.1: Clinical signs of equine grass sickness in the acute, subacute and chronic forms of the disease (adapted from Pirie, 1998).

Clinical signs	Category of EGS		
	Acute	**Subacute**	**Chronic**
Colic	Occasionally	As disease progresses	Mild
Reduced gut motility	Ileus (stasis)	Reduced motility	Reduced motility
Colonic impactions	+	+	-
Small intestinal distension	+	-	-
Gastric reflux	+	As disease progresses	-
Abdomen	Distended	"Tucked up"	Markedly "tucked up"
Dysphagia	+	+	+/-
Tachycardia	+	+	Slightly elevated heart rate
Hypersalivation	+	-	-
Ptosis	+	+	+
Muscle tremors	+	+	+
Patchy sweating	+	+	+
Depression/somnolence	+	-	-
Weight loss	-	+	+
Rhinitis sicca	-	-	+
Base narrow stance	-	-	+

Horses with the acute form of the disease are depressed, tachycardic and exhibit hypersalivation. On rectal examination small, firm faecal pellets with varying amounts of mucus are present.

The clinical signs in the subacute form of the disease are similar but less severe than those seen in the acute form. There is reduced gut motility with colonic impactions. As the disease progresses horses exhibit a rapid loss of weight and condition with the development of a tucked-up abdomen. This is particularly marked in the chronic form of the disease, where the exaggerated tucked up abdomen has been likened to that of an emaciated greyhound (Edwards, 1987; Gilmour, 1988; Pinsent, 1989) (Fig. 1.1). The tucked-up abdomen is not entirely due to loss in body weight. Other characteristics of chronic grass sickness are a base narrow stance, rhinitis sicca and the presence of little or no gut contents on rectal examination. Ptosis (drooping of the eyelid), muscle fasiculations and patchy sweating can also be seen in all three forms of the disease.
Subclinical cases are thought to occur but have not been proven (Pinsent, 1989; Doxey et al, 1995a; Milne, 1997).

There is no single pathognomonic sign for grass sickness, either clinical or biochemical (Doxey et al, 1991a). Biochemical investigation of the blood reflects anhydraemia (Greig, 1942; Doxey et al, 1991a). Serum vitamin E levels and vitamin B12 levels are within normal ranges in grass sickness suggesting the disease affects thriving animals (Doxey et al, 1991a). Diagnosis is based on clinical signs and confirmed at postmortem by the histological examination of peripheral autonomic ganglia for the neuronal degenerative changes characteristic of grass sickness.

Figure 1.1: A horse with chronic grass sickness, showing the characteristic tucked up abdomen and base narrow stance.

Treatment of the disease

There is currently no treatment for horses with acute or subacute grass sickness and animals are euthanased on humane grounds. Previously it was thought that horses with chronic grass sickness very rarely recovered and that if they did they were not capable of work (Greig, 1942; Pinsent, 1989). However, it has been demonstrated that with appropriate treatment, the mainstay of which is thorough nursing care, selected cases of chronic grass sickness can recover and be capable of work (Milne et al, 1994; Doxey et al, 1995b; Milne, 1997; Doxey et al, 1998). Horses with milder symptoms, in particular with respect to dysphagia, appetite, colic, gut sounds and rhinitis, have a greater chance of survival (Milne et al, 1994) and can be selected for treatment on these grounds. The recovery rate from chronic grass sickness at the Large Animal Hospital at the Easter Bush Veterinary Centre was shown to be 48% of all chronic cases entering the hospital, with a recovery rate of 70% in cases selected for treatment (Milne, 1997). The time taken for recovery is variable (Doxey et al, 1998; Doxey et al, 1999), but it has been found that the majority of recovered horses regain weight and return to work within a year (Doxey et al, 1998); 81% of competitive horses returned to their original work.

The horses are encouraged to eat appetising, easily swallowed high-energy food, are groomed, kept dry and warm and their interest stimulated by human contact (Milne, 1997). The prokinetic drug Cisapride, was shown to be of some therapeutic benefit in the treatment of selected cases of chronic grass sickness (Milne et al, 1996). It is an indirect cholinergic agent that facilitates the release of acetylcholine from the postganglionic nerves of the myenteric plexus in the gut. However, it has not been routinely used in the treatment of horses with chronic grass sickness and

has recently been withdrawn from the market. Probiotics have also been used but their benefits have not been scientifically evaluated.

There has been some controversy as to whether horses that have recovered from chronic grass sickness had been correctly diagnosed. Ante-mortem diagnosis is based on clinical signs, and confirmed at postmortem by histopathological investigation of the peripheral autonomic ganglia. Histopathological investigation of ileal biopsies collected at laparotomy can be used to confirm an antemortem diagnosis (Scholes et al, 1993b). However, this procedure requires anaesthesia and surgery, and is considered to reduce the chances of recovery in a horse with chronic grass sickness that is being considered for treatment (Doxey et al, 1995b). In 63 consecutive cases of chronic grass sickness diagnosed clinically antemortem and subsequently euthanased, all were confirmed histologically at postmortem (Doxey et al, 1995b), suggesting that diagnosis by experienced clinicians based on clinical signs is indeed accurate. Recently, recovery from chronic grass sickness has been confirmed in four horses by histological examination of ganglia after their death from other causes several years after recovery (Doxey et al, 2000). There was severe depletion of enteric neurons in all four horses to the extent that it was surprising that two of the horses had not suffered from repeated colic. The other two horses had suffered from intermittent colic after recovery from chronic grass sickness and their ileum showed hypertrophied walls suggesting that the intestinal musculature had adapted to make up for the neuronal deficit. The findings demonstrated the ability of these horses to survive with a significant enteric neuronal deficit.

1.1.2 Neuropathology

Equine grass sickness is a primary dysautonomia with severe neuronal degeneration and loss primarily seen in the autonomic and enteric nervous systems. The characteristic neuronal lesions of grass sickness were first observed by Obel (1955) in the vertebral and prevertebral ganglia and the alimentary mural plexi of the autonomic nervous system. Subsequent investigations of the neuropathology of grass sickness have demonstrated the presence of the neuronal lesions in the central nervous system (CNS): in specific brainstem nuclei, and within the spinal cord, in the dorsal root ganglia, the intermediolateral nucleus and ventral horn (Barlow, 1969, Gilmour, 1973a). Neuronal degeneration in the CNS is not as widespread as that seen in the autonomic nervous system, lesions appear to be restricted to specific nuclei. Equine grass sickness can thus be described as a polyneuropathy as neuronal degeneration occurs in the peripheral, central and enteric nervous systems (Cottrell et al, 1999).

A wide variety of neurotransmitter systems are affected in equine grass sickness. Neuronal degeneration is observed in both the parasympathetic and sympathetic ganglia. Increased amounts of noradrenaline have been observed in intraganglionic swellings (Gilmour, 1976). There are increased levels of circulating adrenaline and noradrenaline in horses with grass sickness, although this might be due to overactivity of the adrenal medulla initiated by stress as much as to overactivity of the central and peripheral sympathetic nervous systems (Hodson et al, 1984a). The enteric cholinergic neurons from the ileum of horses with grass sickness exhibited altered cholinergic mechanisms with a reduction in the release of acetylcholine (Murray et al, 1994). Noncholinergic nonadrenergic neurotransmitters also appear to be affected. There are abnormalities of the regulatory peptide system of the gut

with a marked reduction in the numbers of nerve fibres staining for vasoactive intestinal polypeptide (VIP), substance P, bombesin and enkaphalin in each layer of the gut (Hodson et al, 1982). No peptidergic neurons could be found in some areas of the gut. Extensive degeneration was observed with degranulation and formation of multiple vacuoles in the P-type nerve fibres (Hodson et al, 1982). Electron microscopy has demonstrated the almost lack of neuronal vesicles in the enteric neurones, except those thought to be adrenergic (Hodson and Wright, 1987).

However, a mixture of abnormal and normal neurons is seen in immediate proximity suggesting that there may be some selectivity in the neuronal damage process.

Neuronal degenerative changes

The affected neurons have a characteristic "chromatolytic" appearance (Obel, 1955). Chromatolysis is the term used to describe the loss of Nissl substance (stacks of rough endoplasmic reticulum). Light microscopy shows that in the early stages of degeneration the perikarya of the affected neurons are rounded and moderately swollen, and later become shrunken and irregular; the nuclei are commonly eccentric and pyknotic (Pollin and Griffiths, 1992). The observation of these degenerative changes in peripheral autonomic ganglia is diagnostic for equine grass sickness. As the disease progresses the autonomic ganglia contain fewer neurons (Pollin and Griffiths, 1992).

Ultrastructural investigation has identified that the earliest recognisable changes affect the rough endoplasmic reticulum (RER) and Golgi (Pollin and Griffiths, 1992). A variety of changes have been observed in the RER: it can have an unusual distribution, with dispersion of individual cisternae throughout the cytoplasm, the

cisternae can be distended, or in some cases the RER is unrecognisable and cells may have a marked increase in smooth endoplasmic reticulum (Pollin and Griffiths, 1992). Most cells no longer have a recognisable Golgi complex; ultrastructural and histological investigations have demonstrated that loss of the Golgi is an early event (Griffiths et al, 1993). Affected neurons can also have increased numbers of mitochondria, lysosomes, dense bodies and autophagic vacuoles (Pollin and Griffiths, 1992). Marked abnormalities have been observed in neuronal cytoskeletal, cytoplasmic and secretory proteins thus various proteins with different functions, localisations and synthetic pathways are affected in the neuronal cell (Griffiths et al, 1993).

Neuronal degeneration and clinical outcome – a toxic aetiology?

The pattern of neuronal degeneration observed in equine grass sickness was thought to reflect a toxic insult. However, the nature and distribution of the neuronal damage in equine grass sickness is unlike that previously observed for any known neurotoxin (Gaskell, 1987). The damage to the enteric nervous system is thought to be the primary event in grass sickness (Hodson and Wright, 1987; Pogson et al, 1992; Doxey et al, 1995a). There is evidence that neuronal destruction and loss had occurred in the jejunum of horses with acute grass sickness prior to cell loss in the peripheral ganglia (Pogson et al, 1992).

The clinical severity of the disease correlates with the extent of the enteric neuronal damage (Scholes et al, 1993a). Neuronal degeneration was observed in the acute cases at most sites investigated along the length of the GI tract whereas in chronic cases it was restricted to the terminal small intestine, particularly the ileum (Scholes et al, 1993a; Doxey et al, 1995a). It has been hypothesised that equine grass

sickness could be caused by an ingested or enterically produced neurotoxin (Pogson et al, 1992; Doxey et al, 1995a). The damage in the ileum is usually severe and extensive irrespective of the clinical differences in the disease and it has been proposed that the ileum could be the main site of entry for a putative ingested neurotoxin (Scholes et al, 1993a; Doxey et al, 1995a). Histological examination of transmural ileal biopsies can be used as an ante-mortem diagnostic tool for confirmation of equine grass sickness (Scholes et al, 1993b).

Dosage and duration of exposure to the putative toxin are thought to be important in the differences in enteric damage seen between chronic and acute cases (Pogson et al, 1992; Doxey et al, 1995a). It is proposed that acute cases are exposed to large amounts of toxin present in both the jejunum and ileum over a short period of time resulting in massive damage. Horses with the chronic form of the disease have less neuronal damage due to exposure to smaller amounts of toxin present only in the distal ileum over a longer period of time. Horses that recover from chronic grass sickness may be exposed to very small amounts of toxin with recovery occurring only when the remaining neurons are able to operate effectively (Doxey et al, 1995a). Exposure to very small doses of toxin could result in subclinical cases; these have been thought to occur during an outbreak of grass sickness (Pinsent, 1989; Doxey et al, 1995a; Milne, 1997).

Damage to the peripheral autonomic ganglia could occur via toxin that has entered the circulation (Doxey et al, 1995a) or by retrograde axonal transport (Griffiths et al, 1994). Investigation of the peripheral ganglia also suggested that more severe neuronal degeneration was found in the acute cases than chronic cases (Gilmour, 1973a; Pogson et al, 1992). Approximately 50% of the peripheral ganglion neuron population was either lost or damaged in horses with acute and chronic grass

sickness (Pogson et al, 1992). Horses with acute grass sickness had extensive neuronal damage but little cell loss, whereas chronic cases had fewer damaged neurons present but lower total counts of neurons. The rate of neuronal loss from the peripheral autonomic ganglia, as opposed to the total number of neurons damaged/lost, was therefore considered a possible factor in determining the severity of the disease (Pogson et al, 1992). Significant differences in the extent of neuronal damage in the peripheral autonomic ganglia between acute and subacute cases were not observed despite clinical differences in the severity of disease (Doxey et al, 1992); chronic cases did have significantly less damage in the peripheral ganglia.

1.1.3 Similar dysautonomias in other species

Primary dysautonomias with clinical, pathological and epidemiological similarities to equine grass sickness have been described in other species: cases of dysautonomia in cats, dogs, hares and rabbits have been described since 1982 (Key and Gaskell, 1982; Rochlitz and Bennett, 1983; Whitwell, 1991; Whitwell and Needham, 1996). The striking similarities between these dysautonomias would suggest a potential common aetiological agent (Pollin and Griffiths, 1992). However, no causative agent has yet been described for any of these dysautonomias.

Feline dysautonomia

Feline dysautonomia was first reported in 1982 by Key and Gaskell. Prior to this the disease appears to have been unrecognised. The disease was seen throughout the UK between 1982 and 1986, when there were very few instances of more than one cat in a household being affected (Rochlitz, 1984 cited in Symonds et al, 1995).

However, since 1986 the numbers of cases of feline dysautonomia have declined with the disease appearing to be mainly restricted to outbreaks associated with closed cat colonies (Symonds et al, 1995).

The neuronal degeneration seen in feline dysautonomia is strikingly similar to that seen in grass sickness. However, large complex stacks of smooth parallel membranes are frequently seen in the affected perikarya of autonomic neurons of cats, but these are not seen in equine neurons (Sharp et al, 1984). These stacks may originate from the cell's Golgi complex.

There are some differences in the clinical symptoms of dysautonomia between species: some horses with dysautonomia are tachycardic and show patchy sweating, whereas almost 50% of cats with feline dysautonomia are bradycardic; horses with acute grass sickness drool saliva, whereas cats and dogs with dysautonomia have very dry mucous membranes; fixed dilatation of the pupils is a striking feature in feline dysautonomia but seldom occurs in grass sickness. The majority of clinical signs in feline and canine dysautonomia suggest that parasympathetic dysfunction is occurring (Pollin and Griffiths, 1992). Grass sickness was said to be a "sympathicotonia" (Greig, 1928). It was recognised that this was not necessarily due to a primary sympathetic stimulation but could also be caused by a depression of the parasympathetic system. However, the picture is considered more complex than this as the classical concept of antagonism between the parasympathetic and sympathetic branches of the autonomic nervous system is now known not to be appropriate for all body systems (Cottrell et al, 1999). In addition, nonadrenergic noncholinergic neurotransmitters have been recognised in the regulation of gut motility (Malone et al, 1999). The difference in clinical signs

seen between the dysautonomias is thought to be due to interspecies differences (Gaskell, 1987).

Leporine dysautonomia

Leporine dysautonomia was first recognised in two hares found dead on an estate on which cases of equine grass sickness had occurred (Whitwell, 1991). Gross, histological and ultrastructural findings were reminiscent of grass sickness (Whitwell, 1991; Griffiths and Whitwell, 1993). Chromatolytic neurons were found in the autonomic ganglia and in brain stem nuclei. The cisternae of the rough endoplasmic reticulum were distended and the Golgi apparatus was not recognisable in affected neurons (Griffiths and Whitwell, 1993). However, stacks of parallel smooth membranes and large lectin-staining cytoplasmic inclusions were seen in some diseased neurons; these are not seen in grass sickness (Griffiths and Whitwell, 1993), but similar stacks are often seen in feline dysautonomia (Sharp et al, 1984). Besides these small differences, the similarities between leporine and equine dysautonomias were thought to be sufficient as to indicate exposure to a common causal agent (Griffiths and Whitwell, 1993).

Rabbit mucoid enteropathy (RME)

Rabbit mucoid enteropathy (RME) has been recognised for many years but the cause is unknown. Recently it has been demonstrated that RME is a dysautonomia after identification of chromatolytic neurons in autonomic ganglia, brain nuclei and the spinal cord in an outbreak in "fancy" rabbits in Norfolk (Whitwell and Needham, 1996). Caecal dysbiosis is a feature of RME, and was hypothesised to result from sudden caecal hyperacidity, with the consequence of overgrowth of bacteria such as *Escherichia coli* and *Clostridium* species (Lelkes and Chang, 1987). RME has the

potential for being a good model for the study of dysautonomia (Whitwell and Needham, 1996).

1.1.4 Epidemiology

The epidemiology of equine grass sickness has been extensively investigated in an attempt to identify an aetiological agent, risk factors and ways of preventing the disease. Many of the observations from the early investigations have been confirmed by more recent epidemiological studies that have used a case-control methodology.

Grass sickness has been associated with grazing ever since the disease was first recognised, hence the name it was given (Tocher et al, 1923, Pool, 1928; Greig, 1942). This association has been confirmed more recently by epidemiological studies of the disease (Gilmour and Jolly, 1974; Doxey et al, 1991b; Wood et al, 1998). It has been shown that there is a reduced incidence of the disease in horses that are even part-stabled (Gilmour and Jolly, 1974). Traditionally, horses used in farming were turned out part time at the beginning of May, with full time grazing commencing at the end of May (Greig, 1942). The majority of cases of grass sickness were seen from May to July with a particularly high incidence of the disease in June (Greig, 1942); cases were occasionally but rarely seen in stabled horses or during the winter months. Although grass sickness can occur in any month there is a definite seasonal occurrence with the majority of cases occurring between April and July (Doxey et al, 1991b; Gilmour and Jolly, 1974; Wood et al, 1998). The older literature reports a predominance of the acute form of the disease in the early summer months, with a subsequent increase in the number of subacute and chronic cases as the season progresses (Pool, 1928; Greig, 1942).

Grass sickness is associated with particular premises, with the disease more likely to occur on premises where grass sickness has previously occurred (Wood et al, 1998), particularly if the disease has occurred within the previous two years (Gilmour and Jolly, 1974). There is an increased risk of the disease in horses that have been on a particular premises for less than two months (Gilmour and Jolly, 1974), and in horses that have recently changed pasture (Greig, 1942; Wood et al, 1998). One study found that 50% of cases had a pasture change in the prior four weeks and 20% of the cases in the prior two weeks (Wood et al, 1998).

Warm dry weather with ground frosts were thought to be climatic conditions predisposing to outbreaks of the disease, whilst wet weather was associated with a decrease in incidence of the disease (Tocher, 1924; Pool, 1928; Greig, 1942). However, investigation of meteorological records could not correlate any particular weather condition with the occurrence of the disease (Greig, 1942). In eastern Scotland, it was found that the disease was associated with cooler drier weather with ground frosts occurring at irregular intervals (Doxey et al, 1991c). The study emphasised the local nature of Scottish weather, making the association of meteorological data with outbreaks of the disease difficult. In a study involving the whole of the UK, 66% of the cases were found to occur after predominantly dry weather (Wood et al, 1998).

Scotland is thought to have a higher incidence of grass sickness than England, with more cases occurring in the east of the country than the west; there is no statistical data to support these observations (Doxey et al, 1991c). The differences in regional occurrence may be due to climatic differences, with the eastern weather pattern predisposing to outbreaks of the disease (Doxey et al, 1991c). Climatic differences may influence vegetation. The incidence of the disease is particularly low in August,

a factor which could be related to August having the highest rainfall in eastern Scotland (Doxey et al, 1991b; Doxey et al, 1991c).

There is a significantly higher incidence of the disease in young animals (Doxey et al, 1991b; Wood et al, 1998; Greig, 1942), with two to seven year olds being most at risk (Gilmour and Jolly, 1974). The disease is rarely documented in suckling foals. Thriving animals have also been reported to be more at risk from the disease (Doxey et al, 1991b), but this has not been confirmed by others. There is a lower incidence of the disease in mares (Wood et al, 1998), but no definite differences in susceptibility between breed types (Gilmour and Jolly, 1974).

Prior contact with a previous case of grass sickness has been associated with a ten-fold decrease in likelihood of disease (Wood et al, 1998). This suggests that non-fatal exposure to grass sickness results in resistance to the disease. This resistance may be in the form of an immune response to the aetiological agent, but it could also demonstrate the presence of inherent resistance to the disease in some horses. Grass sickness seems to occur sporadically with often only one horse being affected. Outbreaks do occasionally occur with multiple cases being reported.

Epidemiological studies have implicated an incubation period for grass sickness, which has been estimated at approximately two weeks. Cases of grass sickness were seen in agricultural horses turned out full time to grass at the end of May – a high incidence of the disease occurred 10 to 14 days later (Pool, 1928). Observation of the development of the disease in horses imported from Ireland where the disease is extremely rare has also suggested a two-week incubation period (Doxey et al, 1991c).

Epidemiological investigations have therefore identified that young horses with no previous exposure to grass sickness, having recently changed pasture or being new to the premises and grazing pasture between April to July where grass sickness has previously occurred, will be at increased risk from the disease. However, the sporadic nature of this disease makes it difficult to implement preventative measures such as restriction to grazing, based on these findings.

With respect to the aetiology of the disease, the epidemiological evidence would suggest that the causal agent was related to grazing. Resistance can develop to the disease in older horses, those remaining on a pasture for longer and those in prior contact with the disease. This resistance to grass sickness may be in the form of an immune response to the aetiological agent, thus providing evidence for an infectious aetiology. Climatic conditions may also influence disease occurrence, possibly through effects on vegetation.

1.1.5 Aetiology of EGS

Identification of the aetiology of equine grass sickness is essential to enable both the specific treatment of the disease and its prevention. Many putative aetiological agents have been considered since the disease was first recognised, including poisonous plants, fungi, deficiency disease, chemical toxins, toxins elaborated in the gut and toxin-producing bacteria. The inability to elucidate the aetiological agent of grass sickness has led to re-examination of some of the older hypotheses as well as the investigation of novel agents.

Poisonous plants

Poisonous plants were among the very first putative aetiological agents to be investigated due to the clear association of the disease with grazing. However, no plants that had a toxic effect on horses were identified from pastures where the disease occurred (Tocher et al, 1923). Alsike clover (*Trifolium hybridum*), in particular, had been suspected, but animals that grazed on pasture where this clover was abundant did not succumb to the disease, and extracts of the clover were not toxic when fed to horses (Tocher, 1924; Greig, 1942). A botanical survey in 1927 could not identify any plant common to all pastures where grass sickness had occurred (Greig, 1942). More recently a survey in UK and Patagonia also failed to identify a plant common to all pastures where grass sickness or mal seco had occurred (Robb et al, 1997a).

Festuca argentina, a plant known to be toxic to animals, was consistently found in the diet of nine horses suffering from mal seco (Uzal et al, 1996). However, attempts to reproduce the disease by feeding the plant to three horses over a 28-day period, failed to produce either the clinical symptoms or pathology of grass sickness.

Fungi

Fungi, growing on the plants on pasture-land, were also considered as potential causes of grass sickness. It was noted that the weather conditions that predisposed for grass sickness also suited the spread of parasitic fungi (Wilson cited in Pool, 1928). Cultures of fungi from fields with grass sickness were tested on horses but failed to reproduce the disease (Greig, 1942). Subsequent work has isolated fungi from horses with grass sickness, but not any one particular species was found to be

associated with the disease (Doxey et al, 1990). It was considered that an endophytic fungus of the plant *Festuca argentina* might have been responsible for mal seco and that the conditions during the feeding experiment were not suitable for elaboration of toxin by this fungus (Uzal et al, 1996). However, endophytic fungi have been identified in fewer than 20% of plants examined from fields where grass sickness and mal seco have occurred (Robb et al, 1997a). Endophytic fungi were not found on all pastures with a history of the disease.

Fusarium species were found on all the pastures investigated in the UK and Patagonia where grass sickness/mal seco had occurred (Robb et al, 1997a). Fusaria have also been shown to out-compete other fungi under dry weather conditions, bringing together the association of weather patterns and outbreaks of grass sickness (Robb et al, 1997a). *Fusarium* species can produce mycotoxins that have been demonstrated to be cytotoxic in vitro to equine sympathetic chain ganglion cells, lung and liver cells (Robb et al, 1997b). However, dosing of horses in Patagonia with *Fusarium* cultures, originally isolated from fields where the disease had occurred, did not reproduce the clinical signs of the dysautonomia nor the histopathological changes (Uzal and Robles, 1997). It has been hypothesised that fusarial toxins might act as a predisposing factor to grass sickness, as mycotoxins are known to have detrimental effects on the immune system, vitamin utilisation and can alter intestinal flora (Robb et al, 1997a).

Altered plant biochemistry and oxidative stress

The role of oxidative stress in horses with grass sickness is currently being investigated. Neurons are particularly vulnerable to oxidative stress, which can result from an excess of free radicals or from depletion in antioxidants (McGorum et

al, 1998). It has been hypothesised that grass sickness is associated with the ingestion of plants that are under metabolic stress due to adverse weather or fungal colonisation (McGorum et al, 2000). Plants collected from fields immediately after a case of grass sickness had altered biochemical composition, decreased antioxidant activity and increased prooxidant activity compared to plants collected form the same fields outside the grass sickness season (McGorum et al, 2000). It was considered unlikely that the prooxidant activity of these plants was sufficient to cause the neuronal degeneration seen in the disease. However, it was considered that the metabolically stressed plants could play a role in the development of the disease, either directly through the production of an undetected neurotoxic metabolite, or indirectly through association with a fungus capable of producing a neurotoxin or through the alteration of the intestinal milieu enabling the overgrowth of neurotoxin-producing bacteria.

Toxin-producing bacteria/ invasive bacterium

Clostridium botulinum

In 1919, a large anaerobic bacillus, morphologically and toxigenically resembling *Clostridium botulinum* (*Bacillus botulinus* as it was then called), was detected in anaerobic cultures of stomach and intestines from horses with grass sickness (Tocher et al, 1923). It was also noted at this time that there were similarities between the clinical symptoms of grass sickness and those of classical botulism. In 1924, Tocher reported that he and his colleagues felt that the results of their investigations were sufficient to conclude that *Clostridium botulinum* was the cause of grass sickness.

Tocher (1923; 1924) put forward various pieces of evidence in support of his theory that *C. botulinum* was the cause of grass sickness. The organism had also been isolated form the spleens of horses with subacute grass sickness. The toxin of this organism could be neutralised by antitoxin against known strains of *Bacillus botulinus*. Both the isolated organism and toxin were toxigenic in experimental animals, and said to reproduce the symptoms of "grass sickness" (Tocher et al, 1923). Antitoxin to botulinum type A was found in chronic and recovered horses but not in normal horses or those with the acute form of the disease (Tocher et al, 1923). Tocher considered that grass sickness was an infection with *C. botulinum* with toxin produced in vivo, rather than an intoxication. In support of this view, complement-binding antibodies specific for both botulinum type A and B were detected in horses with the subacute form of grass sickness, but not in normal horses or those immunised with the toxin only (Tocher et al, 1923).

A protection study was carried out using a toxin-antitoxin mixture prepared from known strains. In total, 1433 horses were vaccinated, 961 in 1922 and 472 in 1923, and uninoculated horses were left on each farm as controls, 355 in 1922 and 453 in 1923. In 1922, mortality in vaccinated horses was 2.8% compared to 9.3% in uninoculated controls, and in 1923 the mortality rate in vaccinated horses was 1.5%, compared to 8.2% of the controls (Tocher et al, 1923; Tocher, 1924). The reduction of mortality from grass sickness in the vaccinated horses has been shown to be highly statistically significant ($p<0.0001$) (Wood et al, 1999). However, it appears that Tocher's contemporaries were not convinced by this protection study.

The work of Tocher et al (1923) was the focus for considerable discussion and criticism when it was presented. There was criticism for the use of an antitoxin toxin mixture prepared from human strains of botulinum, rather than the strains isolated

from the grass sickness horses in the protection study. There was also criticism that the organism isolated from the grass sickness horses had not been conclusively identified as *Clostridium botulinum* (Gaiger in Tocher et al, 1923). The organism had not been isolated in pure culture. A major hindrance to Tocher's hypothesis was that at the time botulism was only known as a primary intoxication and not an infection. The production of toxin within a living animal was not considered possible (Anon, 1927; Greig, 1942). There was therefore significant scepticism as to how the organism could cause the disease. It was not until 1976 that a toxicoinfectious form of botulism, infant botulism, was described in humans (Midura and Arnon, 1976).

Tocher was aware that spores of *Bacillus botulinus* could be swallowed and pass through the gut of animals leaving them unharmed, and he therefore thought that there must be a predisposing cause such as a nutritional or metabolic disturbance for example, leading to gastric irritation (Tocher, 1924). However he assumed that the predisposing cause would enable the spores to cross the gut wall and gain access to the tissue (Tocher, 1924), rather than producing the toxin in the gut as is the case in intestinal toxicoinfection.

Tocher's work consequently failed to find support among the veterinarians. Experimental studies demonstrated that the administration of type B toxin to horses by stomach tube induced acute botulism, not grass sickness (Greig, 1942). It was felt that veterinary opinion was justified in concluding grass sickness was not botulism (Anon, 1927).

The role of *C. botulinum* in the aetiology of grass sickness re-emerged in 1994, when it was hypothesised that toxicoinfection with *C. botulinum* type C could

account for both the clinical and pathological features of this disease (J.K. Miller, unpublished).

Other bacteria

A diplostreptococcus was isolated from the central nervous system of an acute case of grass sickness in (Gaiger, 1922, cited in Greig, 1942). This organism was similar to that which had been aetiologically implicated in Borna disease, a neurological disease of horses that shared some clinical similarities with grass sickness (as well as clear differences). However, attempts to reproduce the disease by injection with the organism failed to reproduce grass sickness (Greig, 1942).

Clostridium perfringens type D (*Clostridium welchii* type D as it was then known) was implicated as an aetiological agent in grass sickness in 1934, when toxin was detected in a small number of acute cases (Gordon, 1934). However, attempts to reproduce the disease by administration of the toxin were negative as was a protection study in 1937 that failed to protect against grass sickness (Greig, 1942).

C. perfringens type A has been implicated as the causative agent in a grass sickness-like illness in Colombia, where the disease has been recognised since 1966. The disease was experimentally reproduced, both clinically and pathologically, by inoculation of horses with supernatants of *C. perfringens* type A cultures, isolated from cases of grass sickness (Ochoa and de Valendia, 1978). Horses that had recovered from the disease were also shown to have higher titres of neutralising antibody to the *C. perfringens* enterotoxin, than controls, thus implicating the enterotoxin as the aetiological or complicating agent of the disease (Ochoa and de Valendia, 1978). However, neutralising antibody to the enterotoxin could not be demonstrated in cases of grass sickness in Scotland (Gilmour et al,

1981). There are some clinical and pathological differences between the disease known as grass sickness in Europe and that described in Colombia, and it has been suggested that they may be different diseases.

Other causes

In 1942, a review of the progress in the investigation into the aetiology of grass sickness ruled out the involvement of plant poisoning, a deficiency disease, anaphylaxis, the production of toxic chemicals at pasture, or poisonous products of intestinal origin as causes of this disease (Greig, 1942). Invasive and/or toxin producing bacteria and protozoan parasites were also disregarded as possible causes.

"Filterable viruses" were therefore left as the most likely candidate as the causative agent in 1942. However the nature of this virus or its mode of transmission was unclear. Due to the way in which the disease had initially spread, together with both the epizootic and sporadic nature of the disease, an unwinged motile insect was considered as the most likely vector for transmission. However, an investigation to implicate an insect vector was negative (Greig, 1942) and a virus has never been isolated.

Selenium deficiency was implicated in the aetiology of grass sickness when it was noted that the majority of cases of grass sickness occurred on farms where cattle suffered from muscular dystrophy (MacPherson, 1978). Although selenium levels from horses were reported to be low, so were the selenium levels in healthy animals on the same farm (MacPherson, 1978).

Toxic aetiology

Although the aetiological agent of grass sickness has yet to be identified, it is now generally thought that an ingested or enterically produced neurotoxin is the cause of the disease (Griffiths et al, 1994; Doxey et al, 1995a). Further evidence for a toxic aetiology was acquired when it was discovered that intraperitoneal injection of serum from acute cases of grass sickness could induce the characteristic autonomic lesions of the disease in experimental ponies (Gilmour et al, 1973b), thus demonstrating the presence of a toxic factor in the blood of horses with acute grass sickness.

Inoculation of the ponies with different fractions of the blood identified a plasma protein fraction of molecular weight 30,000Da or greater that was neurotoxic (Gilmour and Mould, 1977). This toxic factor was proteinaceous or was bound to a high molecular weight plasma protein. Later, investigations using thin layer chromatography identified a low molecular weight compound present in extracts of sera from acute cases but not in sera from healthy controls (Johnson, 1985, cited in Pemberton et al, 1990). However, subsequent investigations suggest that this compound was cortisol, a substance found in high concentrations in equine grass sickness (Pemberton et al, 1990). No recognised neurotoxin was detected using chromatography.

However, despite extensive damage to the peripheral autonomic nervous system, in particular the coeliacomesenteric ganglia, there were no clinical signs in the experimental ponies injected intraperitoneally with the acute sera (Gilmour, 1973b). No neuronal lesions were induced by oral administration (Gilmour and Mould, 1977). The absence of clinical signs in these ponies may be due to the fact that the enteric

nervous system was not sufficiently damaged. It is thought that both the peripheral autonomic and enteric nervous systems have to be severely damaged for clinical disease to develop (Pogson et al, 1992; Doxey et al, 1995a).

1.2 *Clostridium botulinum*

The species *Clostridium botulinum* encompasses a phenotypically and genotypically heterogeneous group of organisms, classified as one species on the basis of production of a neurotoxin with the same pharmacological action. The botulinum neurotoxin is one of the most potent toxins known in nature, causing botulism through the inhibition of neurotransmitter release from cholinergic synapses.

1.2.1 Toxin types

C. botulinum has been divided into seven types, A to G, based on the antigenicity of the neurotoxin that they produce. *C. botulinum*, or *Bacillus botulinus* as it was originally named, was first isolated from an outbreak of botulism, in Belgium in 1895, caused by salt-cured, uncooked ham (van Ermengem, 1897 cited in Hatheway, 1989). The toxin types were designated in chronological order of discovery. Botulinum types A, B, E and F are implicated in human cases of botulism whereas types C and D appear to cause only animal botulism. Type G botulinum, first isolated in a soil survey (Gimenez and Ciccarelli, 1970 cited in Hatheway, 1989), has not conclusively been shown to be the cause of botulism in either humans or animals.

The nomenclature of toxin types has been complicated by the isolation of strains that can produce combinations of two distinct toxin types. An isolate from soil

produced 93% type A and 7% type F (Gimenez and Ciccarelli, 1970, cited in Hatheway, 1989) and two strains from children with infant botulism produced 90% type B and 10% type F toxins (Hatheway and McCroskey, 1987), these strains have been designated A_F and B_F respectively. An organism producing a mixture of type A and B toxins has also been identified (Poumeyrol et al, 1983, cited in Collins and East, 1998). Minor variations in toxins produced by different strains of toxin type A (Ciccarelli and Gimenez, 1971, cited in Hatheway, 1989) and toxin type B (Hatheway et al, 1981) have also been discovered. In addition, other species of clostridia have been isolated that are capable of producing botulinum neurotoxin: isolates of *Clostridium baratii* and *Clostridium butyricum* have been identified producing type F and E toxins respectively (Hall et al, 1985; McCroskey et al, 1986).

1.2.2 Phenotypic and genotypic groups of *C. botulinum*

C. botulinum has been grouped into four groups based on phenotypic characteristics (Holdemann and Brooks, 1970; Smith and Hobbs, 1974). Group I contains type A, and proteolytic strains of type B and F; group II contains type E and non-proteolytic/saccharolytic strains of type B and F; group III contains types C and D (usually non-proteolytic); group IV contains type G. The phenotypic characteristics of these organisms are compared in Table 1.2. These groups also contain phenotypically related non-toxigenic organisms.

Genotypic studies, based on nucleic acid hybridisation and 16S ribosomal RNA sequencing studies, have demonstrated the presence of four phylogenetically distinct lineages that agree with the four groups that were based on phenotypic differences (Hutson et al, 1993; Collins and East, 1998). However, comparison of

nucleotide sequences of the botulinum neurotoxin (BoNT) genes does not agree with the four phylogenetic groups of organisms, suggesting that there has been lateral gene transfer of the BoNT genes. BoNT genes are thought to have spread on mobile genetic elements such as transposons, plasmids and bacteriophages: botulinum neurotoxin types E (BoNT/E) and G (BoNT/G) have been found on plasmids (Hauser et al, 1992; Eklund et al, 1988), and BoNT/C and BoNT/D toxins are phage encoded (Eklund et al, 1971; Eklund et al, 1972).

Table 1.2: Phenotypic characteristics of the four groups of *C. botulinum* (I-IV) (Hatheway, 1989)

Group	Type of toxin	Milk digestion	Glucose fermentation	Lipase	Metabolic products[1]		Phenotypically related Clostridium
					Volatile	Non-volatile	
I	A,B,F	+	+	+	A, iB, B, iV	PP	*C. sporogenes*
II	B,E,F	-	+	+	A, B		
III	C,D	+/-	+	+	A, P, B		*C. novyi*
IV	G	+	-	-	A, iB, B, iV	PA	*C. subterminale*

[1] Metabolic acid end products in peptone-yeast extract-glucose medium. A, acetic; P, proprionic; iB, isobutyric;B, butyric; iV, isovaleric; PP, phenylproprionic; PA, phenylacetic.

It has been suggested that *C. botulinum* should be reclassified into four separate species based on these phenotypic and genotypic differences (Hutson et al, 1993; Collins and East, 1998). Species classification based on neurotoxin production alone is both taxonomically incorrect and increasingly complicated. Neurotoxin production is not a stable phenotype in many organisms; other species have been identified as neurotoxin producers, e.g. *C. baratii* and *C. butyricum;* some strains

produce mixtures of two toxin types, and silent BoNT genes have been detected in others. A precedent has been set by the reclassification of *C. botulinum* type G as *Clostridium argentinense*; this organism did not produce lipase, a characteristic of the other *C. botulinum* organisms. Reclassification of the species would enable accurate identification of toxin-producing organisms, important for both the study of the ecology of these organisms and the aetiology of the diseases they cause. The significance of the neurotoxin in disease requires that any reclassification should also account for neurotoxin production by these organisms.

1.2.3 Group III botulinum

C. botulinum type C and D are grouped together by their phenotypic properties into Group III. *C. novyi* type A is phenotypically similar to these organisms and is a non-neurotoxigenic variant of Group III. These organisms cannot be distinguished from each other by traditional methods such as culture, biochemical properties or gas liquid chromatography (GLC) profiles. The surface antigens of *C. botulinum* types C and D and *C. novyi* type A are immunologically cross-reactive (Poxton, 1984; Poxton and Byrne, 1984).

Group III organisms can only be identified to the species/toxin type level by detection of the major toxin produced: type C and D neurotoxins for *C. botulinum* types C and D respectively and the novyi alpha toxin for *C. novyi* type A. These major toxins are each encoded on separate pseudolysogenic bacteriophages. The phage-host relationship is consequently unstable and the phage is readily lost. A cycle of phage loss and reinfection is thought to occur in vivo (Eklund and Poysky,

1974). Repeated subculturing in the laboratory can predispose to loss of the phage. Non-toxigenic Group III organisms are essentially indistinguishable from each other.

On the basis of 16S ribosomal RNA gene sequence analysis the group III strains, including *C. novyi*, are grouped as a separate phylogenetic lineage (Hutson et al, 1993). However, there is some genetic diversity within the Group III strains. There is approximately 99% 16SrRNA sequence similarity between types C and D corresponding to 12 base mismatches and seven unmatched bases over 1500 nucleotides (Hutson et al, 1993). This 1% sequence divergence indicates genetic heterogeneity and suggests that *C. botulinum* type C and D are different species (Collins and East, 1998). There is approximately 38% chromosomal DNA relatedness between these two organisms (Lee and Riemann, 1970). Analysis of 16S rRNA shows that *C. novyi* is also closely related to these organisms with approximately 98% sequence similarity (Hutson et al, 1993); this difference is again sufficient for *C. novyi* to be considered a different species. Analysis of DNA-DNA homology in Group III strains, placed *C. botulinum* type C into two separate genetically related groups and placed *C. novyi* type A into a third group (Nakamura et al, 1983). Consequently if *C. botulinum* was reclassified and Group III became one species, there would still be some genetic diversity within this species. However, the organisms would be phenotypically very similar.

1.2.4 Toxin production in Group III

Toxins produced by C. botulinum type C and D

C. botulinum type C and D can produce up to three toxins: C1 (BoNT/C) or D (BoNT/D) neurotoxin, C2, a binary ADP-ribosylating toxin, and C3, an exoenzyme also with ADP-ribosylating activity. The C3 exoenzyme is encoded on the bacteriophage together with BoNT/C or BoNT/D.

The C2 toxin is located on the bacterial chromosome and is produced by the majority of type C strains and some type D strains, but not by other botulinum toxin-producing organisms. The C2 toxin is produced only during sporulation and not during vegetative growth; the highest titre of C2 toxin is detected in cultures producing the most spores (Nakamura et al, 1978). The toxin has been extracted from the spore coat suggesting it may be part of the spore structural proteins (Yamakawa et al, 1983). C2 toxin production is not governed by the presence of bacteriophages (Eklund and Poysky, 1974). However, it has been proposed that a plasmid may be involved in mediating C2 toxin production as a derivative of a C2 producing type C strain was isolated that no longer produced the C2 toxin (Eklund et al, 1987).

C. botulinum type C was first isolated in 1922 from the fly larvae of *Lucilia caesar* (green bottle fly) obtained from a carcass of a chicken that had died from botulism in the US (Bengston, 1922). It was also isolated from cattle in Australia in the same year (Seddon, 1922). The organisms isolated from the two separate sources were similar enough to be given the same toxin type. However, differences in the ability of antitoxins to cross-neutralise the toxins from the two organisms led to the

designations of subtypes Cα and Cβ (Gunnison and Meyer, 1929). Anti-sera raised against the Bengston strain cross-neutralised the Seddon strain, whereas antisera raised against the Seddon strain only neutralised the homologous toxin. The Bengston strain was designated as Cα and the Seddon strain as Cβ. *C. botulinum* type D was isolated from an outbreak of botulism in cattle in South Africa (Meyer and Gunnison, 1928). Cα strains were said to produce a combination of BoNT/C, C2 and BoNT/D, with type C1 as the dominant toxin, whereas Cβ produced C2; type D cultures were said to produce BoNT/D, BoNT/C and C2 toxins, with BoNT/D being the dominant toxin (Jansen, 1971).

However, it has subsequently been shown that there are common epitopes in types BoNT/C and BoNT/D toxins resulting in cross-neutralisation between the toxins (Oguma et al, 1980; Oguma et al, 1981; Oguma et al, 1982; Oguma et al, 1984) and therefore type C cultures do not produce BoNT/D, and type D cultures do not produce BoNT/C. Different phages govern the production of BoNT/C and BoNT/D (Oguma et al, 1981). The identification of these phages carrying the neurotoxin genes has rendered the subtypes of type C obsolete; a Cβ strain is one in which the converting bacteriophage has been lost. C2-toxin producing strains of both *C. botulinum* types C and D can potentially produce Cβ strains if the converting phage is lost.

BoNT/C and BoNT/D are closely related with 52.2% sequence identity (Sunagawa et al, 1992). The toxins have antigenic sites that are common to both toxins as well as antigenic sites specific for the toxin type (Moriishi et al, 1989; Oguma et al, 1984). BoNT/C and BoNT/D have also been shown to have strain specific epitopes (Moriishi et al, 1989; Oguma et al, 1984). The heterogeneity that exists in the type

C and D neurotoxins has probably arisen from the mutation or recombination of phage genomes that is thought to occur during the cycles of curing and reinfection of type C and D strains in the environment (Sunagawa and Inoue, 1991).

C3 exoenzyme produced by *C. botulinum* types C and D can be divided into two groups based on antigenicity (Moriishi et al, 1993). There is evidence to suggest that the 21.5kbp DNA fragment encoding the C3 exoenzyme is a mobile genetic element (Hauser et al, 1995) with similarities with the site-specific transposon family of Tn554. It is thought that transposable elements may also be responsible for the transfer of other botulinum toxin genes.

Toxins produced by Clostridium novyi

C. novyi is a heterogeneous group of organisms, classified into four types, A to D based on the production of eight different soluble antigens (Table 1.3). Types A, B and D are pathogenic, causing gas gangrene in man and animals, infectious necrotic hepatitis in sheep and bacillary haemaglobinuria in cattle, respectively (Smith, 1975a). Type C, however, does not produce any of the eight soluble antigens and is not known to be pathogenic to laboratory animals. *C. novyi* type A and B both produce the lethal alpha toxin; types B and D produce the lethal beta toxin. The alpha and beta toxins are encoded by separate bacteriophages (Eklund et al, 1976).

Table 1.3: Toxins produced by *C. novyi* types A, B and C and *C. haemolyticum* (Hatheway, 1990)

Toxin	Activity	*Clostridium novyi*			*Clostridium haemolyticum*
		Type A	Type B	Type C	
Alpha	Necrotising; lethal	+	+	-	-
Beta	Lecithinase; necrotising; lethal; haemolytic	-	+	-	+
Gamma	Lecithinase; necrotising; haemolytic	+	-	-	-
Delta	Oxygen-labile haemolysin	+	-	-	-
Epsilon	Lipase	+	-	-	-
Zeta	Haemolysin	-	+	-	-
Eta	Tropomyosinase	-	+	-	+
Theta	Opalescence in egg yolk	-	trace	-	+

C. novyi type D is also considered as a different species – *Clostridium haemolyticum* – due to the fact that it does not produce the alpha toxin and the disease it causes is different from those caused by types A and B (Smith, 1975a). However, despite these differences, the cultural characteristics of types B and D are very similar and they both produce the beta, eta and theta toxins. The soluble cellular antigens of types B and D are identical (Cato et al, 1982). DNA-DNA homology showed that *C. novyi* type D and *C. novyi* type B form a genetically homologous group (Nakamura et al, 1983). On this basis it appears that *C. haemolyticum* should be considered as *C. novyi* type D.

1.2.5 Bacteriophages and Group III

Pseudolysogenic phage and C. botulinum types C and D

Although phages have been identified in Groups I, II and III, they have only been associated with toxigenicity in Group III (Eklund et al, 1989). Production of neurotoxins by *C. botulinum* type C and D is governed by the presence of specific converting bacteriophages (Inoue and Iida, 1970; Inoue and Iida, 1971; Eklund et al, 1971; Eklund et al, 1972). Loss of the bacteriophage is associated with a loss of toxigenicity and sensitivity to reinfection by the converting phage. Non-toxigenic isolates of toxigenic parent strains could be reinfected with the converting phage resulting in a return to toxigenicity. (Eklund et al, 1971; Eklund et al, 1972). Loss of toxigenicity, in the absence of converting phage, was a stable and permanent state (Eklund et al, 1971). The gene for BoNT/C is encoded on a separate phage to the gene for BoNT/D.

These converting phages are thought to be pseudolysogenic because the host-phage relationship is unstable and the phage readily lost from the host cell. Nontoxigenic clostridia resembling *C. botulinum* have frequently been isolated from the environment and toxigenic cultures can become nontoxigenic in the lab (Eklund et al, 1987). This suggests that the phage is not stably integrated into the host chromosome. Bacterial cells can be cured of the converting phage by either ultraviolet irradiation or acridine orange (Eklund et al, 1971); ultraviolet irradiation is a standard method for curing bacteria of lysogenised prophages and acridine orange has been used to remove extrachromosomal elements from bacteria. As both methods cure *C. botulinum* type C and D of the converting bacteriophage, this has been taken as further evidence of pseudolysogeny (Eklund et al, 1971). Subculture of bacteria in the presence of phage specific antiserum also results in

curing, supporting a pseudolysogenic relationship between phage and host (Eklund et al, 1971). Although loss of phage also occurs spontaneously during subculturing, a higher number of nontoxigenic strains are isolated in the presence of anti-phage serum (Oguma, 1976). This suggests that in the absence of anti-phage serum, nontoxigenic strains can be reinfected by free converting phages present in the culture media. Heat treatment of spores of toxigenic strains also resulted in non-toxigenic isolates (Eklund et al, 1972).

Interconversion of C. botulinum type C and D by bacteriophage

C. botulinum types C and D have been shown to be interconvertible (Eklund and Poysky, 1974). Loss of the converting phage from these bacteria renders them indistinguishable from each other. The non-toxigenic derivative strains of *C. botulinum* type C could be infected with a converting phage from a *C. botulinum* type D strain, resulting in production of BoNT/D and essentially changing the non-toxigenic *C. botulinum* type C into a toxigenic type D. The non-toxigenic derivative of a *C. botulinum* type D could also be infected with a type C converting phage resulting in production of BoNT/C. Infection with different specific converting phages effectively changed the toxin type of *C. botulinum*.

Interconversion of toxin types was demonstrated both between strains that were C2 toxin producers and between strains that were not (Eklund and Poysky, 1974). However, in the non-C2 producing strains, the type C strains when cured could be infected by either the type C phage or the type D phage, but the type D strains could only be reinfected by the homologous phage. Cultures infected with type C phages were immune to infection with type D phages and vice versa indicating the antigenically relatedness of the phage (Eklund et al, 1987). It has been proposed

that interconversion between type C and D strains could occur in nature due to the pseudolysogenic state: nontoxigenic strains could be converted to type C or D by different converting phages.

These results suggest that types C and D can arise from a common nontoxigenic strain. However, genetic differences based on the 16S rRNA gene sequence suggests that *C. botulinum* type C and D are different species (Hutson et al, 1993). This observation was based on the comparison of only one strain of *C. botulinum* type C and one strain of *C. botulinum* type D. Therefore it is possible that these genetic differences may also be observed within strains designated type C or type D if more strains were investigated. Genetic heterogeneity has been demonstrated within *C. botulinum* type C strains (Nakamura et al. 1983).

Phages and C. novyi toxins – interconversion of toxin types

The lethal alpha and beta toxins produced by *C. novyi* types A, B and D are encoded by pseudolysogenic bacteriophages (Eklund et al, 1976). A *C. novyi* type B strain that has lost the phage encoding the alpha toxin resembles a *C. novyi* type D strain. A phage has been isolated from a *C. novyi* type A strain that can infect a *C. novyi* type D strain, converting it to alpha toxin production and essentially converting the organism to *C. novyi* type B strain (Schallehn and Eklund, 1980). However, a phage carrying the alpha toxin gene that can infect both type A and B strains has not yet been isolated.

C. novyi type A when cured of the alpha toxin phage continues to produce the gamma and epsilon toxins; *C. novyi* type B when cured of the alpha toxin phage continues to produce the beta toxins (Eklund et al, 1976). Whilst the gamma toxins

and beta toxins are both lecithinases, they are immunologically distinct, moreover, the epsilon toxin is a lipase enabling differentiation between type A and B by the opalescence produced by colonies of the former and not by the latter on egg yolk agar plates. The production of the gamma toxin (lecithinase) and epsilon toxin (lipase) by *C. novyi* type A results in the close resemblance of the cultural characteristics of this novyi type with *C. botulinum* types C and D. In fact the epsilon toxin is shared in common with botulinum types C and D (Hatheway, 1990). Therefore, when *C. novyi* type A loses the phage for the alpha toxin it phenotypically resembles *C. botulinum* type C and D, whereas when *C. novyi* type B lose the phage for the alpha toxin it phenotypically resembles *C. novyi* type D. It has been suggested that *C. novyi* type D is a subtype of type B (Oakley et al, 1947).

Analysis of DNA-DNA homology demonstrated that: *C. novyi* type B and D are genetically more closely related to *C. botulinum* type C than to *C. novyi* type A (Nakamura et al, 1983). *C. haemolyticum* has been shown to have 99.3% 16S rDNA sequence similarity with *C. botulinum* type C and D (Stackebrandt et al, 1999). This suggests that while *C. novyi* type A is phenotypically similar to *C. botulinum* type C, it appears that that *C. novyi* type B and D are genotypically more similar to *C. botulinum* type C.

Species interconversion between C. botulinum and C. novyi by phage

The main phenotypic difference between *C. botulinum* types C and D and *C. novyi* type A is toxin production. Loss of the specific phage carrying the relevant toxin gene by any of these three organisms results in non-toxigenic strains that are essentially indistinguishable from each other. It has been shown that interspecies conversion can also occur between *C. novyi* and *C. botulinum* by infection with the

relevant specific converting bacteriophage (Eklund et al, 1974). A non-toxigenic derivative of a toxigenic *C. botulinum* type C could be infected with a phage from a toxigenic *C. novyi* type A resulting in production of novyi alpha toxin by a strain that was originally producing type C neurotoxin; the nontoxigenic derivative could also be converted to *C. botulinum* type D by infection with a type D converting phage. The phage could be carried through the spore state. When the strain was infected with either the type C or type D phage it was immune to infection by both the type C and D phages but continued to be sensitive to infection with the novyi alpha phage. Consequently the strain could simultaneously produce botulinum and novyi toxins. When the organism was infected both by the novyi phage and the type C or D phage, both phages were often carried through the spore state but one of the phages was frequently lost on subculture (Eklund et al, 1987). On the basis of these findings it has been suggested that a common bacterial strain could exist in nature, with the infecting phage governing toxigenicity and therefore the resulting disease (Eklund et al, 1974).

The parent strain of the nontoxigenic derivative that was used in this interconversion study was isolated from a broiler chicken from an outbreak of botulism and identified as *C. botulinum* type C on the basis of the production of the type C neurotoxin (Roberts et al, 1973). However, the nontoxigenic derivative strain was found to produce the gamma toxin of *C. novyi* type A. On this basis it was thought that the strain involved in the botulism outbreak could have been a *C. novyi* type A strain in nature before being infected with the type C phage; the organism may have produced both the novyi alpha toxin and the type C toxin in nature with loss of the alpha toxin phage occurring before or during isolation and subculture in the laboratory (Eklund et al, 1987).

Antigenicity and stability of phage

The type C and D converting phages, share similar biological and biophysical properties, have similar physiochemical characteristics and close DNA homology and are thought to form a phage family (Sunagawa and Inoue, 1992). However, these converting phages are heterogeneous and have been divided into four groups according to the spectrum of host cells infected and antigenicity of the phage (Oguma et al, 1976; Sunagawa and Inoue, 1991). This classification is similar to that derived from the antigenic structures of the toxin themselves (Oguma et al, 1986). Conversion to toxigenicity therefore occurs only with specific phages and bacterial hosts. Other phages have been isolated from *C. botulinum* type C, D and *C. novyi* type A strains that do not convert nontoxigenic strains to toxin production (Eklund et al, 1987; Sunagawa and Inoue, 1991). Strains of *C. botulinum* type C have been found to carry the converting phage and/or the non-converting phage. The non-converting phages are identical in their antigenicity and host specificity, are smaller than the converting phages and do not determine toxigenicity of the host organism (Sunagawa and Inoue, 1991).

Some strains differ in their stability of toxin production, due to varying instabilities of the pseudolysogenic phage. Loss of toxigenicity is observed at different rates with different combinations of phage and host (Oguma, 1976). It has been shown that the toxigenicity can vary depending on the passage history of the particular phage, with the converting ability of phage decreasing after many propagations through a strain (Oguma and Iida, 1979). Incubation of cultures at 37°C selected virulent mutants of converting phage that lacked the ability to pseudolysogenise bacteria, inducing lysis of their host cells (Eklund et al, 1987).

Strains of type C isolated from areas where avian botulism is enzootic can differ greatly in their toxigenicity (Jensen and Allen, 1960, cited in Wobeser, 1987). Certain environmental conditions may predispose to instability of the phage-bacterium relationship thereby influencing the epidemiology of disease caused by type C botulinum such as avian botulism (Hariharan and Mitchell, 1976). Avian botulism outbreaks rarely occur in areas of high alkalinity but often occur in areas of moderate to low alkalinity such as in the marshes near the mouths of rivers (Kalmbach et al, 1934, cited in Eklund et al, 1987). High salt concentrations inhibit the multiplication of *C. botulinum* type C (Eklund et al, 1987), but low concentrations of salt increase both the rate of infection of *C. botulinum* type C by phage and the stability of this phage–host relationship (Eklund and Poysky, 1974). This may result in the increase in the number of toxigenic *C. botulinum* type C found in peripheral areas of salt flats where a diluted salt concentration is found. These areas are often associated with outbreaks of avian botulism (Eklund et al, 1987).

Flies, maggots and invertebrate carcasses have been implicated in avian botulism and may act as reservoirs of toxigenic bacteria and/or free converting phages capable of converting nontoxigenic strains to toxigenic forms (Hariharan and Mitchell, 1976). Reservoirs of spores carrying converting phages are found in areas where avian botulism is endemic; toxigenic bacteria and phages can also be transferred by migrating birds, winds and floods (Eklund et al, 1987). It has been shown that many different *C. botulinum* type C strains carry phages that are capable of converting strains from geographically distant sources (Eklund et al, 1987), suggesting that this can and does occur in nature. The presence of specific converting bacteriophages, governs the pathogenicity of disease caused by the Group III botulinum organisms. *C. botulinum* types C and D cause botulism through the action of the types C and D neurotoxins respectively, whereas *C. novyi* type A

causes gas gangrene through the action of the alpha toxin. These bacteriophages also define the identity of the organism.

1.3 Toxin action and role in pathogenesis

The catalytic modes of action of BoNT/C, C2, C3 and novyi alpha toxins are well defined at the intracellular level due to their use as biological tools to study neuroexocytosis, the actin cytoskeleton and the involvement of Rho GTPases in eukaryotic signal transduction cascades. However, less is known about these toxins with respect to receptor binding and cell entry. Consequently the role in pathogenesis of disease is less well established than their catalytic action, for some of these toxins.

1.3.1 Botulinum neurotoxin

Botulinum neurotoxins inhibit the release of the neurotransmitter acetylcholine from cholinergic synapses. The neurotoxin is synthesised as a single inactive 150kDa polypeptide chain that is cleaved by bacterial or host proteases to produce the dichain, covalently linked by a single disulphide bond. The dichain is composed of a heavy chain (100kDa) and a light chain (50kDa). Determination of the crystallographic structure of the neurotoxin (type A) has confirmed the presence of three functionally distinct domains (Lacy et al, 1998) that had previously been proposed from biochemical data (Montecucco and Schiavo, 1993) (Fig. 1.2).

Botulinum neurotoxins have been proposed to act by way of a four-step model (Montecucco and Schiavo, 1994; Montecucco, Papini and Schiavo, 1994), involving

(1) cell binding, (2) internalisation, (3) membrane translocation and (4) target modification in the cytosol. Whilst the intracellular action of the toxins has been well characterised, steps one to three are much less well experimentally defined.

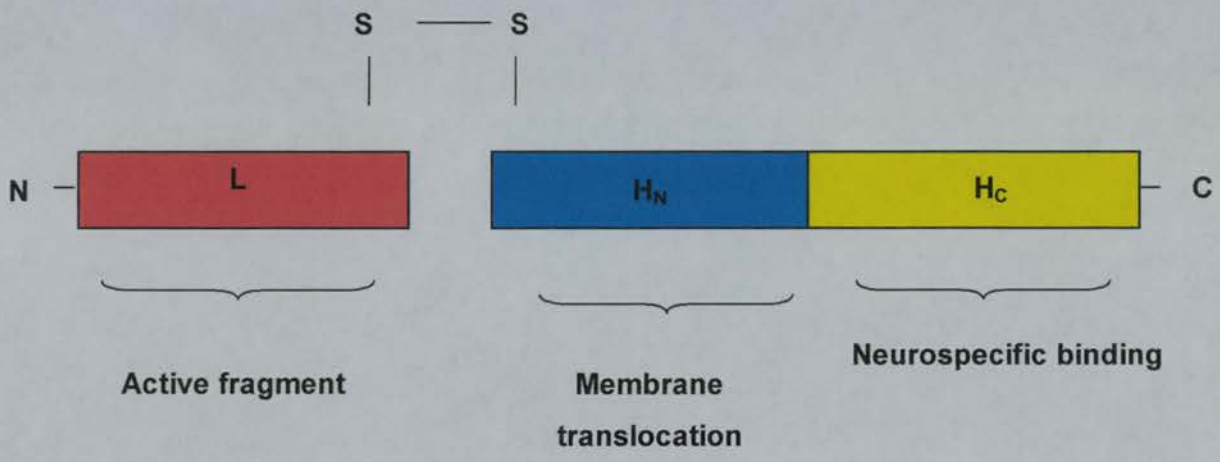

Figure 1.2: Schematic structure of botulinum neurotoxin, showing the three functionally distinct domains (adapted from Oguma et al, 1995).
L=light chain,
H_N = N-terminal end of heavy chain
H_C = C-terminal end of heavy chain.

Progenitor toxin

The neurotoxin is produced in a stable complex termed progenitor toxin. There are three forms of the complexes, ranging in size from 300-900kDa (with sedimentation constants of 12S, 16S and 19S) (Schiavo et al, 2000). BoNT/C and BoNT/D form both 12S and 16S complexes (Ohishi and Sakaguchi, 1980). The 12S complex consists of the neurotoxin and a non-toxic non-haemagluttinin component (NTNH);

the 16S toxin consists of the neurotoxin, a NTNH component and a haemagglutinin component in the ratio 1:1:2 (Fujinaga et al, 1997). The genes encoding the haemagglutinin and NTNH component are encoded immediately upstream of the neurotoxin gene (Hauser et al, 1995).

There is evidence of gene recombination due to the observation of chimera-like or mosaic non-toxic-non-haemagglutinins (NTNH); the phylogeny of the NTNHs is not the same as that of the botulinal toxins despite their genes being contiguous (Collins and East, 1998). The non-toxic components associated with the BoNT/C and BoNT/D are reported to be identical in their antigenicity (Oguma et al, 1980) and nucleotide sequence (Inoue et al, 1999), whilst the neurotoxins are antigenically distinct.

The non-toxic components are thought to be important in the pathogenesis of botulism as they protect the neurotoxin from proteases and acidity in the GI tract (Ohishi and Sakaguchi, 1980). The larger the progenitor complex, the higher the oral toxicity.

Neurotoxin Binding

The most common route of entry for botulinum toxin is via the GI tract, either through ingestion of preformed toxin or production of toxin in the GI tract. There are two stages of binding before botulinum toxin in the GI tract can cause neurotoxicity. First, the toxin must leave the GI tract and enter the circulation, secondly, the neurotoxin must bind to the presynaptic nerve terminal.

Binding to intestinal epithelium

The HA component of the 16S type C progenitor toxin has been shown to bind to glycolipids or glycoproteins in guinea pig small intestine (Fujinaga et al, 1997). It has been shown that 16S type C and D toxins bind to sialylglycolipids and sialoglycoproteins but not neutral glycolipids or asialoglycoproteins (Inoue et al, 1999). Neither the type C neurotoxin, nor the NTNH component bound to the small intestine (Fujinaga et al, 1997). The neurotoxin did not dissociate from the 16S complex in the intestine. It is now thought that the 16S complex has greater oral toxicity than the 12S complex or 7S (neurotoxin alone) due to a higher binding affinity for the intestinal epithelial cells (Fujinaga et al, 1997). The 16S type C complex has been shown to bind to peptidoglycan of certain *C. botulinum* type C strains at pH2 and this cell-bound form was shown to have higher oral toxicity in chickens than the cell-free form (Hyun and Sakaguchi, 1988; Hyun and Sakaguchi, 1989).

Botulinum toxins are thought to cross the intestine by transcytosis (Maksymowych and Simpson, 1998). Human intestinal cells bound and transcytosed BoNT/A and BoNT/B in a functionally active form, but BoNT/C toxin was not efficiently transcytosed. The lack of an efficient mechanism for transcytosis for BoNT/C in human intestinal epithelial cells may explain why type BoNT/C is not associated with human cases of botulism (Maksymowych and Simpson, 1998). However, uncomplexed toxin was used – the 16S form may bind to human cells. Uptake by M cells has not been excluded as a mechanism of entry into the circulation (Schiavo et al, 2000).

Binding to the presynaptic nerve terminal

BoNT/C has been shown to bind to both cholinergic and adrenergic cells in vitro (Yokosawa et al, 1989; Kurokawa et al, 1987). BoNT/C bound with higher affinity to neuroblastoma cell lines of mouse origin compared to those of human origin (Yokosawa et al, 1989). The heavy chain is associated with binding (Yokosawa et al, 1989; Agui et al, 1983).

The C terminal domain of the heavy chain (H_C) has been implicated in neurospecific binding (Schiavo et al, 2000). This C terminal domain is composed of two distinct subdomains H_CN and H_CC. The H_CN is highly conserved between the botulinum toxins and there are structural similarities between this subdomain and carbohydrate binding proteins (Schiavo et al, 2000). The H_CC domain is poorly conserved between the neurotoxins and is required for binding. A double receptor model for binding of neurotoxins to the presynaptic nerve terminal has been proposed, involving a polysialoganglioside receptor that is similar for the different neurotoxins, and a glycoprotein receptor that is specific for the different neurotoxins (Schiavo et al, 2000). Preincubation of botulinum toxins with gangliosides can inhibit binding; the degree of inhibition varies with different gangliosides. GT1b binds to botulinum neurotoxins causing loss of toxicity irrespective of toxin type (Kozaki et al, 1984). However, binding of BoNT/C is also completely inhibited by pre-treatment of cells with neuraminidase, trypsin or pronase implicating a glycoprotein containing sialic acid as a receptor for BoNT/C (Yokosawa et al, 1989).

Internalisation

Internalisation of botulinum neurotoxin into vesicles is temperature and energy dependent (Black and Dolly, 1986). Receptor mediated endocytosis via clathrin

coated pits is proposed to be the mechanism of internalisation (Niemann, 1991). The H_C domain has been shown to be sufficient for internalisation (Schiavo et al, 2000).

Membrane translocation

The light chain has to enter the cytosol to reach its intracellular substrate, and therefore must be able to leave the compartment in which it has been internalised. It is thought that this stage is brought about by acidification of the vesicle, with the low pH inducing a conformation change in the structure of the toxin, enabling penetration of both the H and L chains into the hydrocarbon core of the vesicle membrane. The neurotoxins have been shown to form pH-dependent and voltage-dependent channels in planar lipid bilayer membranes (Donovan and Middlebrook, 1986); the N terminal of the heavy chain is involved in the formation of channels (Blaustein et al, 1987). Translocation of the L chain across the membrane is followed by refolding in the cytosol (Schiavo et al, 2000).

Intracellular action

The botulinum neurotoxins are zinc dependent metalloproteases with the ability to cleave, at specific sites, the protein components involved in neuroexocytosis (Schiavo et al, 1992; Schiavo et al, 1993a; Schiavo et al, 1993b; Blasi et al 1993a; Blasi et al, 1993b). BoNT/C cleaves syntaxin (Blasi et al, 1993b, Schiavo et al, 1995) and SNAP-25 (Williamson et al, 1996; Foran et al, 1996; Osen-Sand et al, 1996). BoNT/D cleaves synaptobrevin/VAMP (vesicle associated membrane protein) (Schiavo et al, 1993b). The light chain contains the zinc-binding motif HEXXH; zinc plays a central role in peptide bond hydrolysis (Schiavo et al, 1993c).

The toxins cleave a single site in their target proteins resulting in sustained blockage of neurotransmitter release.

Syntaxin and SNAP-25 are bound to the presynaptic membrane, and synaptobrevin/VAMP is a synaptic vesicle membrane protein. Together these three proteins form the synaptic SNARE complex, the core of the neuroexocytosis apparatus. These three proteins act as v-SNARES and t-SNARES and interact with the soluble factors NSF (N-ethylmaleimide-sensitive protein), and α/β and γ SNAPS (soluble NSF fusion protein) to bring about membrane fusion and exocytosis (Söllner et al, 1993) (Fig. 1.3). This complex has high structural stability and when assembled, the proteins are resistant to the action of the neurotoxins (Hayashi et al, 1994).

It is thought that the neurotoxins recognise their substrate through a double recognition process; they recognise the substrate by interaction with a region A, a structural motif which is common to all three toxin targets, and region B which contains the peptide bond to be cleaved (Rossetto et al, 1994). This explains the observation that short peptides containing only the cleavage site are not cleaved (Montecucco and Schiavo, 1994), and why only one of several identical peptide bonds in the protein are cleaved by the toxin. The structural motif common to all three toxin targets is the SNARE motif, consisting of nine amino acid residues (Schiavo et al, 2000).

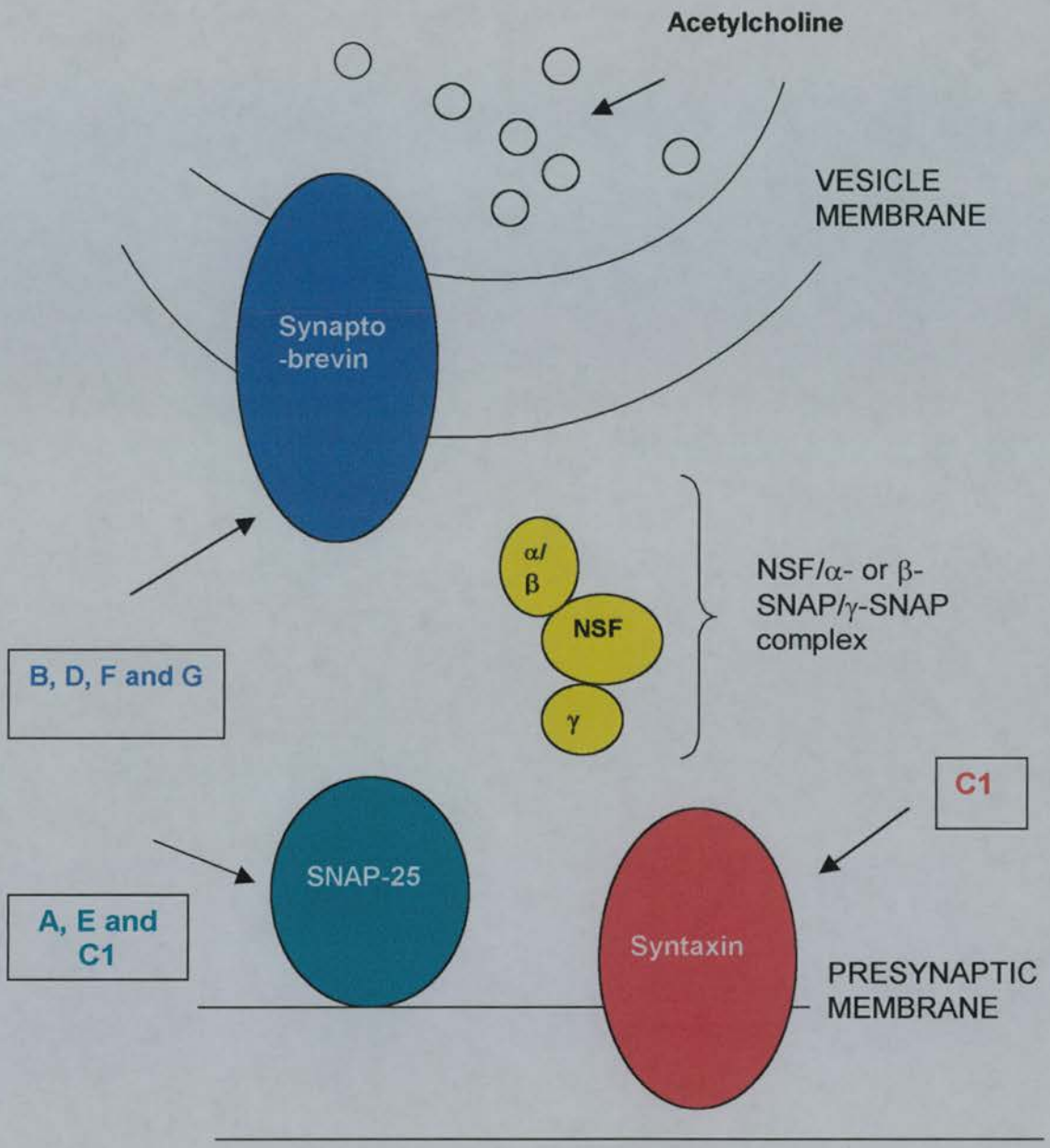

Figure 1.3: Schematic diagram showing the proteins of the proposed neuroexocytosis apparatus involved in vesicle docking and fusion, and the targets of the botulinum neurotoxins (adapted from Oguma et al, 1995).

Cytotoxicity of type C toxin

BoNT/C is severely cytotoxic in vitro to murine spinal cord neurones (Kurokawa et al, 1987; Williamson et al, 1995; Williamson and Neale, 1998), and rat hippocampal and cortical neurons (Osen-Sand et al, 1996). This cytotoxicity is unique to the BoNT/C toxin; the other clostridial neurotoxins block neurotransmission, but only the BoNT/C can cause overt neuronal degeneration (Williamson et al, 1995). BoNT/C is cytotoxic to both mature and developing neurons in vitro (Williamson and Neale, 1998). Primary neuronal cultures incubated with BoNT/C showed a loss of normal structures after 48 hours with accumulation of degenerated mitochondria, membranous dense bodies and vesicles; the neuronal somas were spherical with distorted nuclei and loss of Nissl bodies (Kurokawa et al, 1987). The ultrastructural changes are similar to the axon reaction i.e. retrograde degeneration, in vivo, therefore BoNT/C might disturb axonal flow resulting in degeneration. In mature cultures the synaptic terminals first became enlarged, shortly after exposure to BoNT/C and this was followed by the degeneration of axons, dendrites and cell bodies (Williamson and Neale, 1998). It is thought that the neuronal death is mediated by the effect of BoNT/C on syntaxin in the synapse.

Syntaxin is the intracellular target for BoNT/C but not for the other botulinum neurotoxins. Consequently, BoNT/C has been used to show that syntaxin is essential for neuronal survival and development (Williamson et al, 1995; Kurokawa et al, 1987; Igarashi et al, 1996). The intracellular targets of the botulinum neurotoxin, are conserved within neuronal synapses, and therefore as would be expected, botulinum neurotoxins can inhibit other neurotransmitters besides acetylcholine in vitro (Williamson and Neale, 1998; Mackenzie et al, 1982; Foran et al, 1996; Bigalike and Habermann, 1981; Sugiyama, 1980). It is therefore possible

that BoNT/C can cause damage to a wide range of neurons in vivo. However, the BoNT/C has not been shown to cause loss of motor neurons in humans when used to treat dystonias (Eleopra et al, 1997).

Botulinum neurotoxins in disease

Botulism can occur in three forms: classical botulism and two forms of toxicoinfectious botulism. Classical botulism is caused by the ingestion of preformed toxin, whereas toxicoinfectious botulism results from absorption of toxin that has been produced in vivo following colonisation of the intestinal tract or a wound with *C. botulinum*. This latter form, wound botulism, is relatively rare; the main route of exposure to botulinum toxin is via the GI tract.

Botulinum types A, B, E and F are mainly but not exclusively associated with botulism in humans, while types C and D affect only animals (Rocke, 1993). There have been very few reports of botulism in humans caused by type C: two human cases in USSR in the 1960s (Matveev et al, 1966 cited in Jensen and Price, 1987), a case in France (Meyer et al, 1953 cited in Jensen and Price, 1987) and a case in the US (Prevot et al, 1955, cited in Jensen and Price, 1987). However, these cases were not well substantiated (Holdemann, 1970). It is not known why type C rarely causes botulism in humans. BoNT/C can cleave recombinant human syntaxin and block neurotransmitter release from isolated human neuromuscular junctions (Coffield et al, 1997). It has been proposed that the human GI tract does not have receptors for BoNT/C thereby preventing translocation into the circulation (Maksymowych and Simpson, 1998). However, a lack of exposure to this toxin by humans may also account for the absence of disease caused by this toxin type.

Botulism has been documented in most domestic animals and avian species; Outbreaks of botulism most frequently affect birds (Lamanna, 1987), and *C. botulinum* type C is responsible for the majority of cases of botulism in birds both in the wild and in commercial flocks, despite birds being susceptible to all types of botulinum toxins. Outbreaks of type C botulism have also been reported in mink, ferrets, pigs, cattle, dogs and horses (Mitchell and Rosendal, 1987). There is variability between species in susceptibility to the botulinum toxins; horses are more susceptible to botulism than other species, such as cattle, dogs or humans (Lewis and Metzger, 1980, cited in Whitlock and Buckley, 1997).

Foodborne botulism – Ingestion of preformed toxin

Foodborne botulism is the classical form of the disease and is caused by the ingestion of pre-formed toxin that has been produced in food. The aetiology of botulism was identified in 1895 by van Ermengem after the investigation of an outbreak in Belgium caused by salt-cured uncooked ham (van Ermengem, 1897 cited in Hatheway, 1989). Botulism in humans is relatively rare and has traditionally been associated with the consumption of home-canned/processed low acid vegetables (MacDonald et al, 1986). There were 124 reported outbreaks between 1976-1984 in the US with a mean number of 2.7 cases per outbreak (MacDonald et al, 1986). The disease is more common in animals and this is probably due to an increased consumption of spoiled foodstuffs that would not be consumed by humans.

Rotting carcasses, vertebrate and invertebrate, are one of the most common sources of botulinum toxin for animals and birds. Sarcophagous maggots from these carcasses, containing pre-formed toxin, can be a significant source of toxin for

birds (Mitchell and Rosendal, 1987). Maggots from putrefying carrion have been shown to contain high concentrations of BoNT/C (Haagsma, 1973 cited in Haagsma, 1987). High levels of BoNT/C have been shown to persist over the winter in sarcophagous larvae and pupae of blow flies collected from carrion, providing a source of toxin for a potential outbreak of avian botulism in the spring (Hubálek and Halouzka, 1991).

Death of an animal from botulism without removal of the carcass provides a further source of contamination; *C. botulinum* is thought to invade the tissues from the gut, post-mortem. High levels of BoNT/C were detected for at least 28 days in carcasses of mice incubated at 23°C (Smith and Turner, 1987); the mice had been inoculated with type C spores just before death. Higher levels of BoNT/C and BoNT/D have been experimentally induced in mouse carcasses compared to BoNT/A, BoNT/B and BoNT/E (Ortiz and Smith, 1994a; Smith and Turner, 1987; Smith and Turner, 1988). This may account for the observation that botulism in animals is usually caused by *C. botulinum* types C and D.

Landfill sites have been shown to be a potential source of botulism for gulls that are notorious for their scavenging feeding habits (Ortiz and Smith, 1994b). Spores of *C. botulinum* types B, C and D were each found in 63% of landfill sites examined (Ortiz and Smith, 1994b). It is thought that the gulls themselves are probably responsible for contamination of the landfill sites through the carriage of spores in their alimentary tracts (Ortiz and Smith, 1994b).

In domestic animals, botulism caused by the ingestion of preformed toxin is often called 'forage poisoning' as it is associated with the consumption of contaminated

feed. The source of contamination is often carrion. Forage poisoning in horses is usually due to type C in Northern Europe, whilst in North America it is usually caused by type B. There are subtle differences in the clinical signs of botulism caused by type C as opposed to type A or B (Whitlock and Buckley, 1997; Whitlock, 1996).

Toxicoinfectious botulism

Intestinal colonisation

Toxicoinfectious botulism is caused by intestinal colonisation with *C. botulinum* with production of toxin in the GI tract. Infant botulism was first recognised in 1976 (Midura and Arnon, 1976) and is now the most common form of botulism in the US (Hatheway, 1990). Toxin-producing *C. botulinum* colonises the GI tract of infants less than one year and absorption of the toxin results in flaccid paralysis. It is thought that the lack of an established normal gut flora permits germination of spores and growth of *C. botulinum* in the GI tract. Honey contaminated with spores of *C. botulinum* type B has often been implicated in many cases of infant botulism caused by type B (Arnon et al, 1979).

Intestinal colonisation with *C. botulinum* in adults does occur but is extremely rare and always associated with an altered GI environment, for example after surgery or antibiotic usage (Chia et al, 1986). The presence of *C. botulinum* in the healthy GI tract is transient and *C. botulinum* is not considered to be part of the normal human flora (Dezfulian, 1989). Both in infant botulism and in adult intestinal colonisation the organism and toxin have been demonstrated in the faeces long after the patient has recovered and symptoms gone (Hatheway, 1979; McCroskey and Hatheway, 1988).

'Shaker foal syndrome' is a toxicoinfectious form of botulism that occurs in foals between two weeks and eight months of age, and is usually due to type B. It is thought that spores are ingested from the soil and are able to germinate and colonise the foal's GI tract due to the presence of an immature flora. The toxin is produced in necrotic lesions in the GI tract; high levels of corticosteroids in the mare's milk may play a role in the pathogenesis of this disease (Swerczek, 1980a; Swerczek, 1980b). Unlike in infant botulism, the toxin is only detected in the foal's faeces during the acute clinical phase of the disease. Cases of adult toxicoinfectious botulism in the horse have not been documented (Whitlock and Buckley, 1997).

Toxico-infection with *C. botulinum* type C can occur in outbreaks of botulism in broiler chickens (Eklund et al, 1987). The high-energy diet of these broiler chickens is thought to enhance both the rate and amount of toxin produced in the GI tract (Eklund et al, 1987). Toxigenic and non-toxigenic *C. botulinum* type C strains have been isolated from the poultry litter; this litter is thought to be a source of intestinal infection for the chickens (Eklund et al, 1987). Poultry litter, either fed to cattle or used for the fertilisation of fields, has been the source of outbreaks of type C botulism in cattle (Ortolani et al, 1997; McLoughlin et al, 1988); carcasses and spores present in the litter are the sources of contamination.

Wound botulism

Wound botulism was first identified in 1943 when *C. botulinum* type A was recovered from an infected wound from a patient who had died of flaccid paralysis (Davis et al 1951, cited in Hatheway, 1990). Wound botulism is the least common form of botulism. Only 16 cases were reported between 1976 and 1984 in the US. It occurs when a wound becomes infected with *C. botulinum*, and the neurotoxin that

is subsequently produced enters the circulation causing botulism. The clinical symptoms of the disease are the same as those caused by ingestion of preformed toxin. Recently wound botulism has been associated with IV drug abuse (Macdonald et al, 1985).

1.3.1. Toxins that affect the cytoskeleton

The C2 toxin, C3 exoenzyme and the *C. novyi* alpha toxin all affect the actin cytoskeleton, causing a characteristic "rounding-up" of cells in vitro. Whilst this cytopathic effect is essentially indistinguishable, these toxins are structurally unrelated and functionally have very different mechanisms of action (Figs. 1.4 and 1.5).

C2 toxin

C2 toxin ADP-ribosylates the G-actin form of β/γ cytoplasmic and γ smooth muscle actin (Mauss et al 1990), resulting in the depolymerisation of the actin microfilament network. C2 is a binary toxin composed of two separate components C2I and C2II (Aktories et al, 1992a). However, the functional interaction of these components is required for toxicity. C2I (50kDa) contains the ADP-ribosyltransferase activity; C2II (100kDa) is responsible for binding to the host cell (Ohishi and Miyake, 1985, cited in Aktories and Wegner, 1989).

The C2 toxin belongs to a class of actin-specific mono-ADP-ribosyltransferases that includes the *Clostridium perfringens* iota toxin, *Clostridium spiriforme* toxin and *Clostridium difficile* ADP-ribosylating toxin.

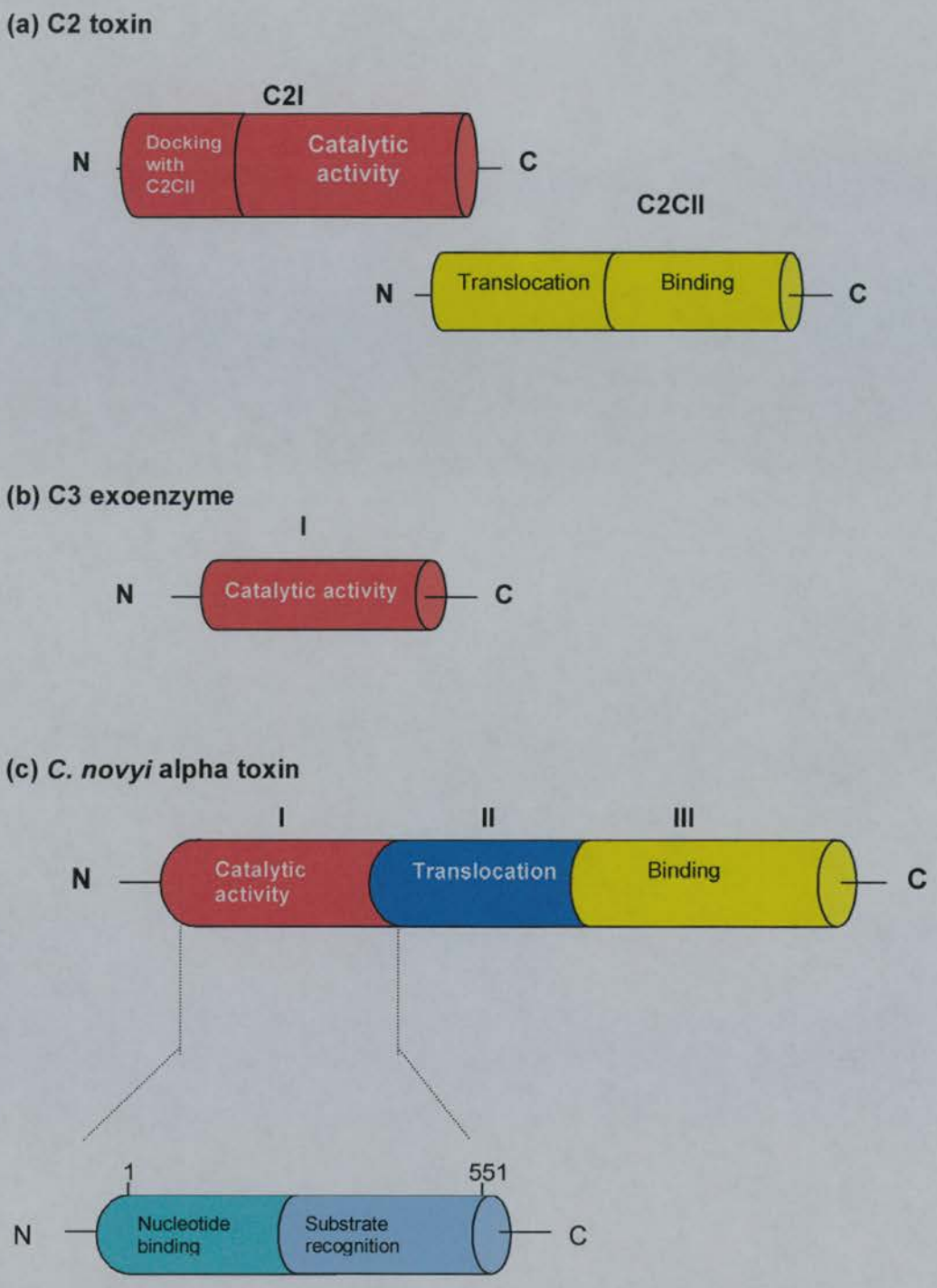

Figure 1.4: Toxins that act on the cytoskeleton. Structural and functional domains of (a) C2 toxin, (b) C3 exoenzyme and (c) *C. novyi* alpha toxin (adapted from Busch and Aktories, 2000; Aktories et al, 2000).

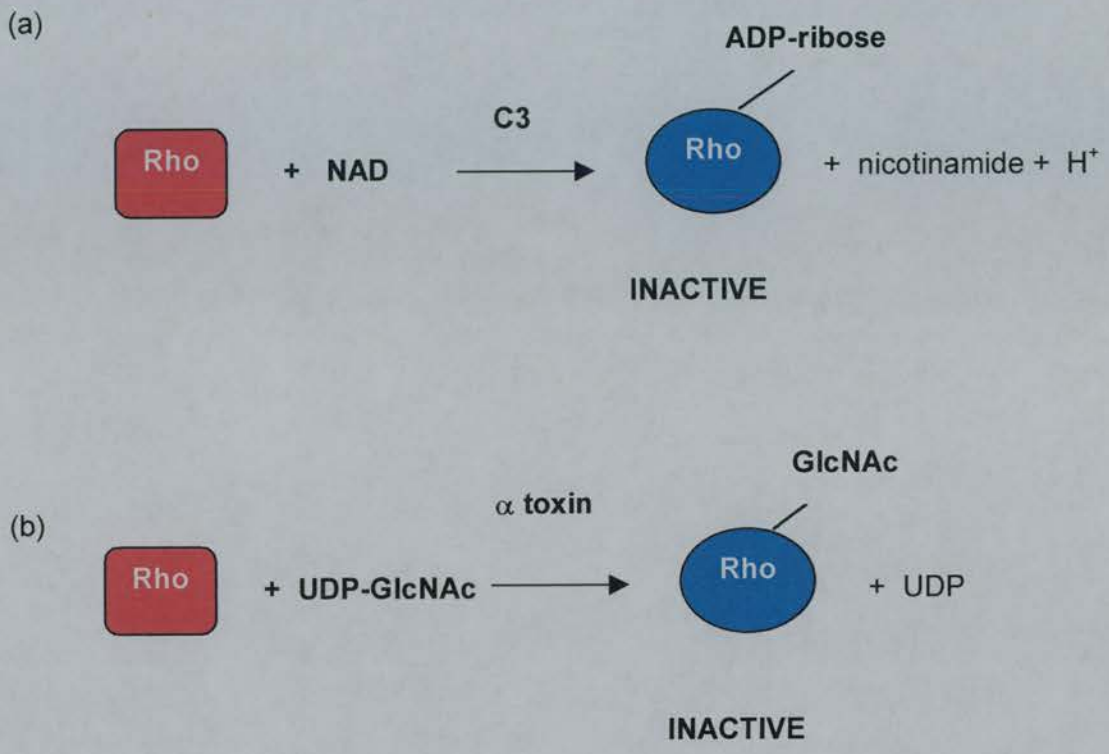

Figure 1.5: Modification of Rho by (a) C3 exoenzyme and (b) *C. novyi* alpha toxin

NAD= nicotinamide adenine dinucleotide
ADP= adenosine diphosphate
UDP-GlcNAc= UDP-N-acetylglucosamine

C3 exoenzyme

C3 exoenzyme is encoded on the same bacteriophages as BoNT/C and BoNT/D (Popoff et al, 1990). The exoenzyme is a separate protein of 25kDa produced together with the neurotoxin (Aktories and Hall, 1989).

The C3 exoenzyme belongs to a group of toxins classified as the C3 exoenzyme family that includes toxins of *Clostridium limosum* and *Bacillus cereus* (Aktories et al, 1992b) and the epidermal differentiation inhibitor (EDIN) produced by *Staphylococcus aureus* (Sugai et al, 1990). The C3 exoenzyme family are all approximately 25kDa in size, with 30-70% amino acid sequence identity and ADP-ribosylate the small molecular mass Rho GTPases (Aktories, 1997).

C3 exoenzyme does not appear to have domains for binding or membrane translocation; purified C3 is not able to enter most intact cells (Aktories and Hall, 1989). Therefore, despite the potent catalytic activity observed in vitro when introduced artificially into a cell, it is debatable whether the enzyme has toxic effects in vivo.

C. novyi alpha toxin

The *C. novyi* alpha toxin has been classified as a large clostridial cytotoxin (LCT); this group also includes *Clostridium difficile* toxins A and B, and the haemorrhagic and lethal toxins of *Clostridium sordellii* (Bette et al, 1991). These toxins are high molecular weight single-chain molecules (250-300kDa) that glycosylate the low molecular weight GTP binding proteins of the Rho subfamily. There is 32-76% amino acid sequence homology between the LCTs (Busch and Aktories, 2000).

The novyi alpha toxin is a 250,166 Da protein (Hofmann et al, 1995) with 48% amino acid sequence homology with the *C. difficile* toxins A and B. A wide range of mammalian cells have been shown to be susceptible to these toxins in vitro. The toxin is thought to be composed of three functional domains: the N-terminal domain involved in catalytic activity, a central hydrophobic domain involved in membrane translocation and a C-terminal domain involved in cell binding (Von Eichel-Streiber et al, 1996).

Cell binding

Binding of C2 toxin

Proteolytic activation of the C2II domain is essential for C2 binding and uptake by cells. Trypsin cleaves a 20kDa peptide from the N-terminus of C2II resulting in an active form that oligomerises, forming predominantly heptamers (Barth et al, 2000). Whole C2II (i.e. not trypsinised) does not from oligomers. The C-terminus (amino acid residues 592 to 721) of the C2II domain is involved in binding to the cell surface (Blöcker et al, 2000); binding of C2II was blocked both by an antibody against the C-terminus and truncation of this region. The site for receptor binding that is formed after oligomerisation of C2II may be stabilised by the seven C-terminal amino acids, as deletion of these residues also blocks binding (Blöcker et al, 2000).

Activated (trypsinised) C2II binds to asparagine-linked complex carbohydrates as determined through the investigation of a CHO (Chinese hamster ovary) cell mutant that was resistant to C2 toxin. The cell line was found to be deficient in N-acetlyglucosaminyltransferase I (Eckhardt et al, 2000). This defect prevented the cells from making N-linked complex and hybrid carbohydrates. Restoration of N-

acetlyglucosaminyltransferase I, through transfection of the gene, made the cells sensitive to the C2 toxin (Eckhardt et al, 2000). The β-1,2-GlcNAc linked to the α-1,3-mannose of the asparagine–linked core structure was found to be essential for the binding of C2II to the CHO cells.

Although there are sequence homologies between the C2 toxin and other ADP-ribosylating toxins, there are no sequence similarities in the C-terminal domain of these toxins indicating different receptor binding. It is not known whether a protein receptor is also required for cell binding. There is evidence for the involvement of a carbohydrate receptor alone; no naturally C2 toxin-resistant cell line has been found suggesting that the C2 receptor is ubiquitously expressed (Eckhardt et al, 2000).

The 225-amino acid N-terminal part of C2I is responsible for the contact of C2I with a docking site on C2II (Barth et al, 1998a). This docking site is formed after binding of the C2II to the cell surface receptor. Construction of a fusion protein of the N-terminal 225 amino acid residues of C2I with a C3-like Rho-ADP-ribosylating toxin from *C. limosum* enabled both translocation and intracellular cytotoxicity when the chimeric protein was applied with C2II to cultured cells.

Binding of C. novyi alpha toxin

Clostridial repetitive oligopeptides (CROPs) have been identified in the C-terminal domain of the large clostridial cytotoxins (Hofmann et al, 1995). CROPs consist of 20-50 amino acids that are repeated 14-30 times and function as ligands, forming multivalent receptor binding domains and enabling multiple interactions with a potential receptor (von Eichel-Streiber et al, 1996). Lectin-like binding has been described for *C. difficile* toxin A, but nothing is known of the receptors for the other

LCTs including *C. novyi* (Busch and Aktories, 2000). However, there is sequence homology between the CROPs of the LCTs (von Eichel-Streiber et al, 1996). The *C. novyi* alpha toxin is thought to act ubiquitously against mammalian cells (Ball et al, 1993) suggesting the presence of a ubiquitous receptor.

Cell entry – Internalisation and membrane translocation

C2 toxin

C2 toxin enters cells by receptor-mediated endocytosis (Simpson, 1989). It is thought that the C2 toxin enters the cytosol from an acidified endosomal compartment. Bafilomycin A1, a fungal metabolite that prevents acidification of endosomal vesicles through the blocking of the vacuolar-type H^+-ATPase in the endosomal membrane, blocked C2 toxicity (Barth et al, 2000). Activated C2II can form ion-permeable channels in artificial lipid bilayer membranes (Schmid et al, 1994). It is thought that after acidification of the early endosomal compartment, oligomeric C2II forms a channel in the membrane thereby mediating translocation of C2I into the cytosol (Barth et al, 2000).

C. novyi alpha toxin

The novyi alpha toxin is also taken up by receptor-mediated endocytosis and is thought to require endosomal acidification to enter the cytosol (Ball et al 1993). Acidification is thought to result in a conformational change in the toxin permitting translocation into the cytosol. The central hydrophobic domain is thought to facilitate insertion of the toxin into the membrane of the endosome and the subsequent translocation process (von Eichel-Streiber et al, 1996).

Intracellular activity

Modification of actin by C2 toxin

C2I is the enzymatic component of the C2 toxin. The C2I component mono-ADP-ribosylates G-actin at arginine 177 resulting in inhibition of actin polymerisation (Aktories et al, 1986). Actin is an essential component of the microfilament network of the cytoskeleton and is also involved in various motile processes such as migration, phagocytosis, secretion and intracellular transport (Aktories and Wegner, 1992). The physiological functions of actin are highly dependent on its capacity to polymerise and form microfilaments. Actin exists in equilibrium between the filamentous form (F-actin) and the monomeric globular form (G-actin) (Aktories and Wegner, 1992). The ADP-ribosylated G-actin acts as a capping protein binding to the ends of actin filaments and preventing further polymerisation; depolymerisation occurs at the opposite end of the "capped" microfilament (Aktories and Wegner, 1989).

Site directed mutagenesis has enabled characterisation of the catalytic site in the C-terminal domain of C2I. Conserved glutamic acid residues at positions 389 and 387, together with serine-348 and arginine-299 were found to be essential for ADP-ribosyltransferase activity (Barth et al, 1998b). A catalytic glutamic acid residue is highly conserved within other ADP-ribosyltransferases (Barth et al, 1998b). Serine-348 thought to be involved through either NAD-binding or catalysis; arginine-299 is also thought to be involved in NAD binding and/or stabilisation of the active site structure. These residues are also conserved in other arginine-modifying ADP-ribosyltransferases from both prokaryotes and eukaryotes.

Modification of Rho proteins by C3 exoenzyme and C. novyi alpha toxin

C3 exoenzyme and *C. novyi* alpha toxin both modify the same target proteins, Rho GTPases, but at different sites and in different ways. The Rho protein subfamily belongs to the Ras superfamily. C3 exoenzyme ADP-ribosylates Rho-A, -B and -C (Sekine et al, 1989), whereas *C. novyi* alpha toxin glycosylates Rac and Cdc42, in addition to Rho (Selzer et al, 1996). Both modifications occur in the effector region of the proteins.

Rho-GTPases regulate the actin cytoskeleton and are involved in various eukaryotic signal transduction processes (Hall, 1998; Bishop and Hall, 2000). The Rho subfamily primarily regulates the organisation of the actin cytoskeleton: Rho is involved in the formation of stress fibres and associated focal adhesions, Rac is involved in the formation of membrane ruffles, lamellipodia and focal contacts, and Cdc42 is involved in the formation of filopodia. Rho proteins also function as molecular switches in signal transduction cascades, thus controlling many cellular responses to a variety of extracellular signals. Rho proteins can regulate migration, adhesion and cell polarity; control transcription, cell cycle progression and transformation; and play key roles in the regulation of secretion, phagocytosis, endocytosis, NADPH oxidase and neurotransmission (Aktories et al, 2000; Dousseau et al, 2000).

The Rho proteins function as molecular switches through nucleotide exchange in the GTPase cycle (Bishop and Hall, 2000) (Fig. 1.6). This cycle is regulated by at least three groups of interacting factors. Rho is bound to GDP in its inactive form and is found mainly in the cytosol. Binding to guanidine nucleotide dissociation inhibitors

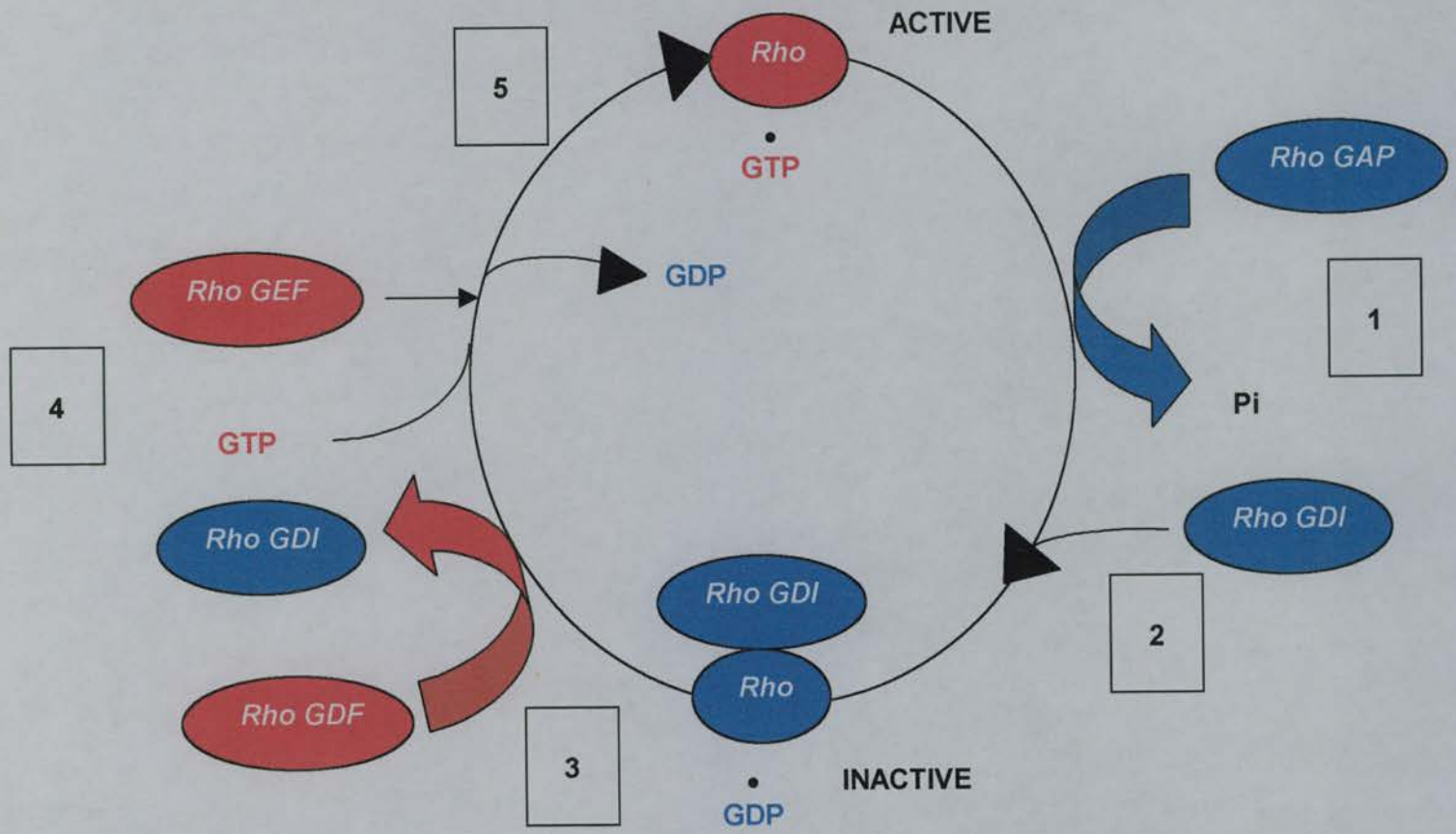

Figure 1.6: The Rho GTPase cycle (adapted from Kaibuchi et al, 1999). Blue represents inhibitory factors and inactive forms of Rho. Red represents activating factors and active forms of Rho.

1. GTPase activating protein (GAP) increases hydrolysis of GTP to GDP, converting Rho to the inactive GDP-bound form.
2. GDP dissociation inhibitor (GDI) binds specifically to Rho in GDP bound form, preventing dissociation of GDP.
3. GDI dissociation factor (GDF) results in dissociation of GDI from Rho, in response to extracellular signals.
4. Guanidine nucleotide exchange factor (GEF) facilitates binding of GTP by Rho, converting Rho to the active GTP bound form.
5. The GTP-bound form is targeted to the cell membrane where it interacts with its specific targets.

(GDI) maintains Rho in the inactive GDP-bound form. Rho is activated by GDP/GTP exchange induced by guanidine nucleotide exchange factors (GEFs). Binding of GTP results in a conformational change in the protein enabling interaction with and activation of effector molecules. Hydrolysis of GTP to GDP returns the protein to its inactive form; this hydrolysis is facilitated by GTPase-activating proteins (GAPs).

The *C. novyi* alpha toxin modifies Rho at threonine 37, only when Rho is in the GDP-bound form; threonine 35 is the homologous amino acid that is modified in Rac and Cdc42 (Selzer et al, 1996). This threonine residue is a conserved amino acid in the effector region of the Rho proteins. It is involved in nucleotide (GDP/GTP) binding by the Rho proteins via the coordination of a magnesium ion. This residue is only accessible to the alpha toxin in the GDP-bound conformation (Aktories, 1997). The alpha toxin catalyses the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to Thr 37/35 of the Rho GTP-binding proteins (Selzer et al, 1996). *C. novyi* alpha toxin is the only LCT to use UDP-N-acetylglucosamine as the preferred co-substrate; the other LCTs use UDP-glucose (Busch et al, 2000a). In the absence of a protein substrate, the alpha toxin acts as a glycohydrolase rather than a glycosyltransferase.

The glycosylation of Rho has multiple consequences: the coupling of Rho proteins with their effectors is blocked, nucleotide exchange and membrane cycling is inhibited and both intrinsic and activated GTPase activity is blocked (Sehr et al, 1998; Busch and Aktories, 2000).

C3 exoenzyme blocks the biological activity of Rho GTPase through ADP ribosylation of Rho at asparagine-41 (Sekine et al, 1989). However, it is not fully

understood how this modification affects the functioning of the protein. The modified residue is also in the effector domain of the protein, but ADP-ribosylation, unlike glycosylation, has only minor effects on the nucleotide binding, effector binding and GTPase activity of Rho (Sehr et al, 1998). ADP-ribosylated Rho exhibits enhanced binding to GDI. Release from GDI is required before activation by GEFs and nucleotide exchange can occur; this may in part explain how ADP-ribosylation affects Rho GTPase function (Aktories et al, 2000).

The catalytic activity of the alpha toxin is located in the N-terminal domain. The N-terminal fragment of 551 amino acids was required for full glycosyltransferase activity (Busch et al, 2000a). Amino acids from 133 to 517 in the alpha toxin are thought to contain the region determining the nucleotide-sugar specificity of this toxin, probably through binding of the sugar moiety of the co-substrate (Busch et al, 2000a). Amino acids towards the C-terminal end of this catalytic domain have also been implicated in binding of the protein substrate (i.e. Rho proteins) (Hofmann et al, 1998). There is a conserved DXD motif in a region of high homology in the catalytic domain of the LCTs; this motif is thought to be involved in binding of the nucleotide sugar via the coordination of a manganese ion (Busch et al, 1998). The LCTs have a general dependency on manganese ions (Busch et al, 2000a). In addition, a conserved tryptophan residue at position 102 is thought to interact with the nucleotide moiety of the co-substrate (Busch et al, 2000b).

The role of the toxins in pathogenesis of disease

C2 toxin

The pathophysiological role of the C2 toxin in natural infection is not really understood, despite the ability of the toxin to cause significant morbidity and mortality in experimental animals.

C2 toxin is a lethal toxin (Ohishi et al, 1980a) that can cause an increase in vascular permeability (Ohishi et al, 1980b; Ohishi, 1983a), and fluid accumulation in intestinal loops (Ohishi, 1983b). This enterotoxic activity is accompanied by histological damage to the epithelial surface of the intestine (Ohishi and Odagiri, 1984). C2 toxin can cause hypotension, haemorrhaging and collection of fluids in the thoracic cavity of experimental animals (Simpson, 1982).

Visceral smooth muscle cells are especially rich in γ-actin and poor in α-actin (Mauss et al, 1990), and therefore represent a potential target for the C2 toxin. It has been demonstrated that C2 toxin inhibits the contraction of longitudinal muscle plexus myentericus preparation of guinea pig ileum (Mauss et al, 1989). It is possible that G-actin/F-actin transition is involved in the regulation of smooth muscle contraction (Aktories et al, 1992a).

C2 toxin may have an effect on cells of the immune system. It has been shown to inhibit migration of neutrophils (Aktories et al, 1992a) and to enhance the production of oxygen free radicals and granule protein release in neutrophils (Norgauer et al, 1988). Stimulation of a premature respiratory burst could facilitate evasion of the immune response. The inhibition of histamine release from mast cells has also been reported (Bottinger et al, 1987, cited in Aktories and Wegner, 1989).

There has been very little documented evidence regarding the isolation of this toxin in animals other than birds (Kinde et al, 1991). C2 toxin has been implicated in some cases of type C botulism in broiler chickens where diarrhoea and enteritis have been observed alongside the neuroparalytic signs of botulism (Ohishi and DasGupta, 1987). C2 toxin was isolated from the intestinal tract of one horse during an outbreak of *C. botulinum* type C intoxication associated with the consumption of processed alfalfa hay cubes in North America (Kinde et al, 1991). Intramuscular oedema was observed in five other horses and possibly indicated the involvement of C2 toxin.

C3 exoenzyme

It is debatable whether the C3 exoenzyme has a role in the pathogenesis of disease as it is not known if the enzyme can enter cells in vivo. However, the C3 toxin has been demonstrated to have effects in vitro that could be potentially significant in disease. C3 exoenzyme has been shown to induce depolymerisation of the actin cytoskeleton in the vicinity of tight junctions, resulting in the redistribution of the peripheral tight junction protein ZO-1 (Nusrat et al, 1991). The functional consequence of this is an increased permeability of the epithelium.

C3 has been shown to cause neuronal degeneration in vitro (Williamson and Neale, 1998). However, high concentrations of C3 are required to observe these effects and the C3 toxic effects are not specific to neurons, as the underlying glial cells are also affected. Neuronal degeneration caused by the C3 toxin is less severe than that caused by BoNT/C toxin and more uniform across cell bodies and processes; BoNT/C toxin causes degeneration that starts at the synaptic terminal (Williamson and Neale, 1998).

C. novyi alpha toxin

The novyi alpha toxin has clearly been implicated in the pathogenesis of gas gangrene, in man and animals. Gas gangrene is an invasive anaerobic infection of the muscle, characterised by profound toxaemia, extensive local oedema, massive death of tissue, and a variable degree of gas production (MacLennan, 1962). Although gas gangrene can be caused by a number of pathogenic clostridia, producing different toxins, *C. novyi* type A has been implicated in approximately one-third of cases (Smith, 1975a).

Colonisation of a wound with *C. novyi* type A, with subsequent toxin production can cause gas gangrene. There is a high mortality associated with wound infections involving *C. novyi*, particularly when accompanied with *Clostridium sporogenes* (Smith, 1975a). The pathology and mortality is due to the lethal and necrotising properties of the alpha toxin. The alpha toxin can damage capillary endothelium causing an increase in capillary permeability (Elder and Miles, 1957) and oedema. Recently *C. novyi* has also been associated with deaths in drug injectors (http://www.iduoutbreak.abelgratis.com, 2000).

The most common disease caused by *C. novyi* in animals is infectious necrotic hepatitis. This disease most commonly affects sheep, and is caused by *C. novyi* type B. Therefore, the beta toxin as well as the alpha toxin plays a significant role in the pathogenesis of this disease.

The use of the alpha toxin as a biological tool to study cellular signal transduction cascades controlled by the Rho GTPases, has implicated the potential of this toxin to affect multiple host systems. It has long been known that the alpha toxin can affect epithelial permeability. However, it is now known that this is probably due to

its effect on tight junctions due to the redistribution of the actin cytoskeleton. The alpha toxin could have the potential to effect the functioning of the immune system due to the dependency of immune cells to respond to extracellular signals.

The large clostridial cytotoxins are implicated in different diseases. *C. difficile* causes antibiotic associated diarrhoea and pseudomembraneous colitis due to the effect of toxins A and B on the colonic mucosa. *C. sordelli* causes wound infections (gas gangrene) and enterotoxaemia through the production of the lethal toxin and haemorrhagic toxin. Although these toxins use a different co-substrate from *C. novyi* alpha toxin, the modification of the target protein occurs at the same site and with the same effect. The lethal toxin of *C. sordelli* differs in that it modifies Rac but not Rho, and in addition modifies Ras, Ral and Rap (Just, 1996, Popoff et al, 1996); the cytopathic effect produced by this toxin is distinguishable from that caused by the others (von Eichel-Streiber et al, 1996).

The differences in disease caused by these toxins may reflect differences in cell receptor binding between the toxins, or may be due to differences in the ecology of the organisms producing the toxins. The pathogenesis of clostridial diseases has traditionally focussed on the involvement of toxins. However, virulence factors of the organisms themselves may also be significant in defining the disease i.e. establishing the difference between an organism that produces toxin in the gut and one that produces toxin in a wound.

Hypothesis and Aims of Thesis

The aetiology of equine grass sickness is not known. This thesis investigates the hypothesis that grass sickness is caused by intestinal toxicoinfection with *Clostridium botulinum* type C, i.e. colonisation of the small intestine with in vivo production of toxin(s).

It is hypothesised that *C. botulinum* type C is either carried in the GI tract of affected animals or is acquired by the ingestion of spores. It is thought that an unknown trigger, perhaps environmental or nutritional, alters the GI environment to allow either overgrowth of *C. botulinum* type C into the small intestine or the germination of recently acquired spores. The subsequent absorption of *C. botulinum* type C toxin(s) is thought to damage the neurons of the enteric and autonomic nervous systems severely, resulting in the characteristic neuronal lesions that are seen in this disease. It is proposed that an immune response, both to *C. botulinum* type C and the type C toxin(s), could play a role in the prevention of equine grass sickness and in recovery from this disease.

To investigate this hypothesis, the aims were as follows:

1) To detect the *C. botulinum* type C neurotoxin (BoNT/C), and BoNT/C-producing organisms in the GI tract of horses with and without equine grass sickness, in order to establish whether toxico-infection with *C. botulinum* type C is associated with the disease.

2) To isolate *C. botulinum* type C from the GI tract of horses with and without equine grass sickness, and to compare these isolates by phenotypic and

genotypic methods to identify strain types specifically related to equine grass sickness.

3) To investigate the role of the immune response to BoNT/C and the surface antigens of Group III clostridia in horses with and without equine grass sickness. To compare specific antibody levels to these antigens in equine sera, intestinal contents, colostrum and milk, in order to identify the extent of exposure of horses to *C. botulinum* type C and to identify a role for an immune response in the prevention of disease.

Chapter Two

Materials and Methods

2.1 Samples

2.1.1 Samples of gastrointestinal (GI) contents for detection of BoNT/C

Equine specimens

Intestinal contents were collected from the distal ileum at post-mortem and rectal faecal samples were collected either antemortem or at post-mortem. Ileum contents were taken from 29 horses with grass sickness (acute, n=12; subacute, n=7; chronic, n=10) and 28 controls (normal, i.e. apparently healthy horses, n=2; a horse which had grass sickness in the past but not at the time of sampling, n=1; clinical non-grass sickness cases with gut dysfunction, colic n=1, and clinical non-grass sickness cases without gut dysfunction n=15; and with unknown diagnoses, n=9). Faecal samples were taken from 45 horses with grass sickness (acute, n=13; subacute, n=6; and chronic, n=26) and 77 controls (normal, i.e. apparently healthy horses, n=24; horses which had grass sickness in the past but not at the time of sampling, n=2; healthy horses in contact with clinical grass sickness cases, n=34; clinical non-grass sickness cases with gut dysfunction, colic n=4, ileus n=1, and clinical non-grass sickness cases without gut dysfunction, n=11). Horses with grass sickness were diagnosed and categorised clinically as described previously (Doxey et al, 1991a; Doxey et al 1992). The disease was subsequently confirmed in acute, subacute and fatal chronic grass sickness cases by necroscopy, including histopathological examination of autonomic and intestinal ganglia (Doxey et al, 1992).

Specimens from other animals

Intestinal contents were also collected from other animals with dysautonomia: 18 cats (16 cats with dysautonomia and two cats with diarrhoea that were in contact with 13 of the cats with dysautonomia), one hare and two wild rabbits.

2.1.2 Serum samples for the detection of systemic IgG

Blood was collected from 80 horses with grass sickness: 28 with acute grass sickness (AGS), 21 with subacute grass sickness (SGS) and 31 with chronic grass sickness (CGS). Fourteen of the horses with CGS were sampled more than once. Blood was collected from 142 horses that did not have grass sickness. These horses were sub-divided into three groups: 'contacts' (n=60), 'high-risk' (n=46), and 'controls' (n=36). The 'contact' group were horses that had been directly in contact with a case of grass sickness i.e. co-grazing at the time of disease onset. The 'high risk' group were horses grazing pastures considered to be a high risk for grass sickness i.e. where grass sickness had occurred frequently in the past. The 'controls' were horses with no known contact with grass sickness i.e. grazing pastures where grass sickness had not been reported. Of the 36 controls, 17 were healthy animals and 19 were clinical cases, other than EGS (ten were cases associated with GI dysfunction and nine horses were non-GI cases). Six of the healthy controls were sampled at intervals over a 28-week period.

2.1.3 Samples of equine GI contents for the detection of specific IgA

Samples of ileum contents were collected at post-mortem and faecal samples were collected either ante-mortem or post-mortem. Faecal samples were collected from 26 horses with grass sickness: 15 horses with CGS, four horses with SGS and seven horses with AGS. Faecal samples were collected from 48 horses without

grass sickness. Faecal samples were collected from ten horses with CGS on more than one occasion – eight horses were sampled twice, one horse was sampled three times and one horse was sampled six times, over the course of the disease. Faecal samples were collected from six healthy horses three times over the course of a 12-week period – at week one, week four and week 12. Ileum contents were collected from 20 horses with EGS: five horses with CGS, six horses with SGS and nine horses with AGS. Ileum contents were collected from 12 horses without grass sickness.

2.1.4 Gut tissue for antibody extraction and lymphocyte isolation

Sections of small intestine were collected at post-mortem for the isolation of lymphocytes and handled as described in section 2.5. Tissue samples of Peyer's patches or lamina propria, for extraction of antibodies with saponin, were separated from the muscle layers, cut into small pieces and aliquotted into eppendorf tubes. The samples were stored at -70°C until treated with saponin.

2.1.5 Colostrum and milk samples

Colostrum

Colostrum samples were collected up to 12 hours post-parturition from 36 mares, 22 of which had been in contact with grass sickness and 14 of which had not been in contact with EGS. "In contact" was defined as grazing land where grass sickness had previously occurred. Of the 22 mares "in contact", six had been co-grazing with a case of grass sickness i.e. grazing at the time the disease occurred; 14 mares were grazing fields after grass sickness had occurred on these fields; two mares had recovered from chronic grass sickness. The mares involved in this study were of a variety of breeds and were widely distributed throughout Scotland and England.

Milk

Milk was collected at two and four weeks post-parturition and then at four weekly intervals, until weaning, from 36 mares. Twenty-three of the mares had been in contact with EGS and 13 had not. Of the 23 mares "in contact" with EGS, seven were co-grazing with a case of EGS, 14 were grazing fields after a case of EGS had occurred on those fields, and two had recovered from CGS.

Colostrum/milk samples (5-10ml) were collected by the owner into sterile plastic universals and sent by first class post. Both colostrum and milk samples were received from 35 mares; only colostrum was received from one mare and only milk from another.

All samples were labelled with the letter M (for milk study) and each mare was assigned a number. Each sample also received a letter as a suffix, to identify how many weeks post-parturition the sample had been collected - the letter A represented a colostrum sample, B milk collected at two weeks, C for milk at four weeks, D for milk at eight weeks etc. Therefore, M2A represents the colostrum sample from mare number two, and M16E, represents the milk sample collected at 12 weeks from mare number 16.

2.2 Treatment of samples for toxin detection

2.2.1 Processing of samples for direct detection of BoNT/C

For direct detection of BoNT/C, weighed specimens (1-10g) were infused into a known volume (5 or 10ml, depending on consistency of sample) of phosphate buffered saline, pH 7.2, with 0.2% gelatin, (PBSG), and held overnight at 4°C. After

vortexing, the liquid portion was removed and centrifuged at 3,800g for 20 min. The supernatant was subsequently collected and stored at -20ºC.

2.2.2 Processing of samples for detection of BoNT/C after enrichment

For detection of BoNT/C after enrichment approximately 1-3g of specimen was added to 15ml pre-reduced CMC-1 medium [developed by Centre for Applied Microbiological Research, (CAMR), Porton Down, Salisbury, Wiltshire, UK] and incubated anaerobically at 30ºC for 5 days. The samples were centrifuged at 3,800g for 20 min and the supernatant was collected and stored at -20ºC.

2.3 Treatment of samples for detection of antibodies

2.3.1 Serum samples

Blood samples were collected in sterile vacutainers with no additives. The blood samples were allowed to clot overnight at 4°C, and the serum collected after centrifuging at 1000g for 15 min. Serum was stored at -20°C until assayed.

2.3.2 Treatment of GI contents with protease inhibitors

In order to detect IgA present in ileum contents and faecal samples, the samples were treated as soon as possible after collection to inhibit intestinal proteases and preserve the IgA, using a method adapted from Gaspari et al (1988). Samples not treated within 24 h of collection were excluded from this study. The reagents used in treating the samples were all stored at -20°C, and kept on ice during the treatment procedure. Two parts protease inhibitor solution (soybean trypsin inhibitor 1mg/ml in PBS, and 50mM EDTA containing 0.05% Tween 20) were added to one part of weighed sample (approximately 5g) (v/w). Phenylmethlysulphonyl fluoride (PMSF;

Sigma-Aldrich Company Ltd, Poole, Dorset, UK) (0.1M in 100% ethanol) was then added to a final concentration of 1mM. The mixture was vortex mixed for 30-60 sec and then centrifuged at 3800g for 10 min. The supernatant was removed, and PMSF (0.1M in 100% ethanol) was added to the supernatant to a final concentration of 2mM, followed by sodium azide (20mg/ml) to a final concentration of 1% (v/v). The supernatant was mixed well and left to stand for 15 min on ice. Heat inactivated foetal calf serum (FCS) was then added to a final concentration of 4% (v/v). The sample was transferred to microcentrifuge tubes and centrifuged at 15,700g for 5 min. The supernatant was removed and stored at -70°C.

2.3.3 Treatment of samples with saponin

Samples of GI tissue were treated with saponin to extract antibodies. Initially, samples were thawed for different lengths of time at 4°C in PBS containing 2% saponin (Sigma), soybean protease trypsin inhibitor (1mg/ml; Sigma), ethylenediamine-tetraacetic acid sodium salt (EDTA; 0.05M), Tween 20 (0.05%), PMSF (2mM), sodium azide (0.2mg/ml) and 4% FBS; 2ml of solution was added per gram of tissue. The samples were centrifuged at 15,700g for 5 min and the supernatants collected and stored at -70°C until analysed.

2.3.4 Treatment of colostrum and milk samples

Samples were centrifuged at 3,800g for 20 min and the supernatant transferred to microcentrifuge tubes. The samples were then centrifuged at 15700g for 5 min in a microcentrifuge. The supernatant was removed and stored at -70°C until assayed.

2.4 ELISAs

2.4.1 Coating of ELISA plates

ELISA to detect BoNT/C

A sandwich ELISA for the detection of BoNT/C had been developed by CAMR, Porton Down. A polyvalent guinea pig antiserum (supplied by CAMR) raised against the purified neurotoxin was used as a capture. Nunc Immuno™ Polysorp™ ELISA strips (Fisher Scientific UK, Loughborough, Leicestershire, UK) were coated (100µl/well) overnight at 4°C with 5-10µg/ml of the antiserum diluted in PBS pH 7.4. Plates were washed three times with PBS containing 0.1% Tween 20 (PBS-T).

ELISA to detect antibodies to EDTA-extracted surface antigens of *C. novyi* type A and *C. botulinum* type C

The surface antigens of *C. novyi* type A and *C. botulinum* type C were extracted as described in Chapter 2.8.4-2.8.5, except that a bulk culture of 1 litre was used for the extraction. The protein concentration was determined using the Folin protein assay (Lowry et al, 1951).

Nunc Immuno™ Polysorp™ plates were coated with EDTA-extracted surface antigens *of C. novyi* type A (MPRL 2530) or *C. botulinum* type C (MPRL 4240) at 30µg/ml in coating diluent (0.05M sodium bicarbonate buffer, pH9.6 containing 0.02% sodium azide), as described by Poxton (1984); 100µl was added to each well. Plates were incubated overnight at room temperature. After overnight incubation, plates were washed four times with ELISA wash buffer (PBS containing 0.05% Tween 20 and 0.05% sodium azide, pH7.2).

ELISA to detect antibodies to BoNT/C

Nunc Immuno™ Polysorp™ plates were coated with purified BoNT/C (CAMR) at 5μg/ml in PBS overnight at 4°C; 100μl was added to each well. The coating concentration was determined by CAMR, Porton Down. Plates were washed three times with PBS-T.

ELISA for total IgG

Preliminary assays were carried out to establish the optimal dilution for the capture antibody. Two different rabbit anti-equine IgG (H+L) antisera (Nordic Immunology, Tilburg, Netherlands, UK and ICN Pharmaceuticals, Ltd, Basingstoke, Hampshire, UK) were compared. Nunc Immuno™ Polysorp™ plates were coated with anti-equine IgG antisera diluted in ELISA coating diluent (100μl/well). Plates were incubated overnight at 4°C and then washed four times with ELISA wash buffer.

On the basis of the results of these preliminary assays, (Chapter 7.1.1) plates for further assays were coated with rabbit anti-equine IgG (H+L) antiserum (ICN) diluted 1 in 2500.

ELISA for total IgA

Preliminary assays were carried out to establish the optimal coating dilution of anti-equine IgA antibody. Two different antibodies were compared: anti-equine IgA (Fc) rabbit antiserum (Nordic Immunology) and anti-equine IgA mouse monoclonal [Bristol Veterinary School (BVS), Bristol University, UK]. Nunc Immuno™ Polysorp™ plates were coated with anti-equine IgA antibody diluted in coating diluent (100μl/well). Plates were incubated overnight at 4°C and then washed four times with ELISA wash buffer.

On the basis of these preliminary assays (Chapter 7.1.2) plates were coated with anti-equine IgA mouse monclonal (BVS) diluted 1 in 1000

2.4.2 Blocking of ELISA plates

ELISA to detect BoNT/C

Plates were blocked with PBS-T containing 5% foetal bovine serum (PBS-TF; 200µl/well). Plates were incubated, with shaking, for a minimum of 1 h at 37ºC and then washed three times in PBS-T. Plates were stored at -20° until use.

ELISAs to detect total IgG, total IgA, and specific antibodies

Plates were blocked with PBS containing 3% teleostean gelatin (Sigma) and 0.02% sodium azide (200µl/well). Plates were incubated, with shaking, for four hours at 37°C and then washed four times with ELISA wash buffer and stored at -20°C until use.

However, the blocking conditions differed for the ELISAs to detect antibodies in serum, both to BoNT/C and *C. novyi* type A surface antigens: plates coated with BoNT/C were blocked with PBS-TF (200µl/well) and incubated for 1 h, with shaking, at 37°C; plates coated with *C. novyi* type A surface antigens were not blocked.

2.4.3 Addition of samples

ELISA to detect BoNT/C

Sample supernatants were assayed in duplicate at three dilutions (undiluted, 1 in 2, and 1 in 4 for direct toxin detection; 1 in 5, 1 in 25 and 1 in 125 for enriched

samples). Samples were diluted in PBS-TF and 100µl added to each well. The plates were incubated at 37°C, with shaking, for 90 min and then washed three times with PBS-T.

ELISA for antibodies to surface antigens and to BoNT/C

Serum samples

Previous work had determined the optimum dilution as 1 in 50 for the screening of serum samples for IgG both to *C. novyi* type A surface antigens and to BoNT/C (R. Brown and H.G. Lough, personal communication). Samples were diluted in antiserum/conjugate diluent (0.05M phosphate buffer, pH7.4, 0.85% sodium chloride, 0.05% Tween 20 and 0.02% sodium azide), and 100µl was added to each well. Plates were incubated for 90 min at 37°C and then washed four times with ELISA wash buffer.

Other samples

Preliminary assays were carried out to establish the optimum dilution for screening GI contents, colostrum and milk samples for specific IgA both to *C. novyi* type A surface antigens and to BoNT/C. All samples were diluted in antiserum/conjugate diluent, and 100µl added to each well. Plates were incubated overnight at room temperature and then washed four times with ELISA wash buffer.

ELISA for total IgG

Preliminary assays were carried out to establish the optimal dilution for screening samples (colostrum, milk and saponin-treated tissue-extracts) to determine total IgG content. All samples were diluted in antiserum/conjugate diluent and 100µl added to

each well. Plates were incubated overnight at room temperature and then washed four times with ELISA wash buffer.

ELISA for total IgA

Preliminary assays were carried out to determine the optimal dilution of samples (colostrum, milk and saponin-treated tissue-extracts) for the calculation of total IgA. Samples were diluted in antiserum/conjugate diluent and 100µl added per well. Plates were incubated overnight at room temperature, and washed four times.

2.4.4 Primary detecting antibody

ELISA for IgA to surface antigens and BoNT/C

Anti-equine IgA mouse monoclonal [Immune Systems Ltd. (ISL), Paignton, UK] was used as the primary detecting antibody in the ELISAs for detecting specific IgA. Preliminary assays were carried out to determine the appropriate dilution of this antibody. The antibody was diluted in antiserum/conjugate diluent and 100µl added to each well. Plates were incubated, with shaking, for 5 hours at 37°C and then washed four times with ELISA wash buffer.

On the basis of the results of these preliminary assays (Chapter 6.1.1), the anti-equine IgA mouse monoclonal was used at a dilution of 1 in 200.

ELISA for total IgA

Preliminary assays were carried out to compare different anti-equine IgA antibodies for use as a primary detecting antibody in an ELISA to detect total IgA and to establish the most appropriate dilution for use. Rabbit anti-equine IgA (Fc) antiserum (Nordic Immunology), anti-equine IgA mouse monoclonal (Bristol

antiserum (Nordic Immunology), anti-equine IgA mouse monoclonal (Bristol Veterinary School), and anti-equine IgA mouse monoclonal (ISL) were used as primary detecting antibodies in combination with different capture antibodies. The primary detecting antibody was diluted in antiserum/conjugate diluent and 100µl added to each well. Plates were incubated, with shaking, for 3 h at 37°C and then washed four times with ELISA wash buffer.

On the basis of the results of these preliminary assays (Chapter 7.1.2), the rabbit anti-equine IgA (Fc) antiserum (Nordic) was used as a primary detecting antibody at a dilution of 1 in 400.

2.4.5 Conjugate

ELISA to detect BoNT/C

The anti-BoNT/C guinea pig IgG (supplied by CAMR), conjugated to horseradish peroxidase (HRP) was used at a dilution of 1 in 300 in PBS-TF (100µl/well). Plates were incubated for 90 min at 37°C and then washed three times in PBS-T.

ELISA for total and specific IgG

Rabbit anti-horse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was diluted 1 in 10000 in antiserum/conjugate diluent and 100µl added to each well. The plates were incubated, with shaking, for 90min at 37°C and then washed four times with ELISA wash buffer.

ELISA for specific IgA

Goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was diluted 1 in 5000 in antiserum/conjugate diluent and 100µl added to each well. Plates were incubated overnight at room temperature and then washed four times with ELISA wash buffer.

ELISA for total IgA

Goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) or goat anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma), depending on the host species of the first antibody, was used. The conjugate was diluted 1 in 5000 in antiserum/conjugate diluent, 100µl/well added and incubated, with shaking, for 90 min at 37°C. Plates were washed four times with ELISA wash buffer. On the basis of preliminary assays, it was decided to use the rabbit anti-equine IgA (Fc) antiserum (Nordic) and therefore, the goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was used in further assays for total IgA.

2.4.6 Substrate

ELISA to detect BoNT/C

The substrate (3, 3', 5, 5'-tetramethyl-benzidine dihydrochloride tablets, Sigma) dissolved in phosphate-citrate buffer pH5.0 with 2µl 30% H_2O_2 per 10ml, was added to the plate (100µl/well). The reaction was allowed to develop at room temperature for 30 min or until the background became detectable. The reaction was stopped by the addition of 50µl/well 2M H_2SO_4. The absorbance was measured at 450nm, reference at 620nm, in an Anthos plate reader.

ELISA for total and specific IgG and IgA

Alkaline phosphatase substrate tablets, p-nitrophenyl phosphate (Sigma 104), were dissolved in substrate solvent (0.05M sodium carbonate solution, pH 9.8, with 1mM magnesium chloride) to give a concentration of 1mg/ml. The substrate solution was added to each well (100μl/well) and incubated at room temperature. The incubation times varied between the assays: 30 min for total IgG, total IgA, and specific IgG in colostrum and milk samples; 1 h for specific IgG in serum samples, specific IgA in GI contents, colostrum and milk samples. The absorbance was measured at 405nm, reference 620nm, in an Anthos 2001 plate reader.

2.4.7 Controls

ELISA to detect BoNT/C

Purified BoNT/C, diluted in PBS-TF to 100ng/ml, 20ng/ml, 4ng/ml and 0.8ng/ml was included as a standard to enable comparison between plates and quantification of results.

Every third column was left uncoated and received only PBS (100μl/well) during the coating stage (all wells were blocked). Each sample was therefore added to two coated wells and one uncoated well. The uncoated well was used to control for non-specific binding of the sample to the plate. One row of wells received no samples, only PBS-TF (100μl/well), as a negative control.

ELISA to detect specific IgG to surface antigens and BoNT/C

Serum samples

A serum sample had been previously identified that gave an optical density (OD) reading, after dilution, of approximately 1.0 in the assay to detect IgG to *C. novyi* type surface antigens. This serum sample was used as the positive control for both the ELISA to detect IgG to *C. novyi* type A surface antigens and to BoNT/C. The positive control was used to standardise OD readings between plates.

Every third column, in each plate, was left uncoated and received only PBS (100μl/well; BoNT/C coated plates) or coating diluent (100μl/well; *C. novyi* type A coated plates) during the coating stage. Therefore, each serum sample was added to two coated wells and one uncoated well. It was thought that the uncoated well would control for non-specific binding of the serum samples to the plates. One row of wells received no serum samples, only antiserum/conjugate diluent (100μl/well), as a negative control.

Colostrum and milk samples

A positive control was identified by preliminary assays and this was included on each plate to allow standardisation of OD readings between plates. Four or more wells received no samples, only antiserum/conjugate diluent (100μl/well), as the negative control.

ELISA to detect specific IgA

GI contents

A positive control was identified by preliminary assays and this was included on each plate to allow standardisation of OD readings between plates.

In preliminary assays, every third column was left uncoated and received only coating diluent (100μl/well) during the coating stage. Each sample was added to four coated wells and two uncoated wells. The uncoated wells were to control for non-specific binding of the samples to the plate. Two of the coated wells and one of the uncoated wells in the next stage, received no primary detecting antibody, only antiserum/conjugate diluent (100μl/well). This was to control for non-specific binding of the conjugate to the samples. As there was no significant non-specific binding of either the samples to the plate or conjugate to the samples, these controls were not included in further assays. One row of wells received no samples, only antiserum/conjugate diluent (100μl/ well), as the negative control.

Colostrum and milk samples

A positive control was identified by preliminary assays and this was included on each plate to allow standardisation of OD readings between plates.

Initially, some samples were added to four wells; two of these wells did not receive primary detecting antibody, only antiserum/conjugate diluent (100μl/well), in order to control for non-specific binding of the conjugate to the samples. However, as there was no significant non-specific binding of the conjugate to the samples this control was not included in further assays. Four or more wells, received no samples, only antiserum/conjugate diluent (100μl/well), as a negative control.

ELISA to detect total IgG

Dilutions of equine IgG, of known concentration, were added in duplicate (100μl/well) to each plate in order to obtain an equine IgG standard curve. This standard curve was then used to calculate the concentration of IgG in colostrum and

milk samples and saponin-treated tissue extracts. The equine IgG standard was prepared from an equine serum sample (LH 346C). This serum sample was put through a protein G column (Mab Trap GII kit; Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Buckinghamshire, UK) and the IgG fraction was eluted and collected. The protein concentration of this IgG fraction was determined using the Folin protein assay, and this was taken to represent the IgG concentration in the standard. Preliminary assays were carried out using known concentrations of this equine IgG standard to establish the optimal dilutions to obtain an IgG standard curve. Incubation times and conditions were as described for the samples in this assay (Chapter 2.4.3). Based on the results of these preliminary assays (Chapter 7.1.1), the IgG standard was diluted twofold between 0.1μg/ml and 0.003μg/ml. Four or more wells on each plate received no samples, only antiserum/conjugate diluent (100μl/well), as the negative control.

ELISA to detect total IgA

Dilutions of a colostrum sample, of known IgA concentration, were added in duplicate (100μl/well) to each plate in order to obtain an equine IgA standard curve. This standard curve was then used to calculate the concentration of IgA in colostrum, milk samples and saponin treated tissue extracts. The IgA concentration of the colostrum sample was calculated using a commercially available radial immunodiffusion kit (LLA; VMRD Inc., Pullman, WA 99163, USA). Preliminary assays were carried out using known concentrations of this colostrum sample to establish the appropriate dilutions to obtain an IgA standard curve. The incubation times and conditions were as described for the samples (Chapter 2.4.3). Based on the results of these preliminary assays (Chapter 7.1.2), the IgA standard was diluted two-fold between 10 μg/ml to 0.31 μg/ml in antiserum/conjugate diluent.

Initially, some samples (colostrum, milk and saponin-treated GI extracts) were added to four coated wells; two of these wells did not receive the primary detecting antibody, only antiserum/conjugate diluent (100μl/well), to control for non-specific binding of the conjugate to the sample. However, as there was no significant non-specific binding of the conjugate to the samples, this control was not included in further assays. Four or more wells received no samples, only antiserum/conjugate diluent (100μl/well), as the negative control.

2.4.8 Calculation of ELISA results

BoNT/C levels in GI samples

The mean OD for the negative control was subtracted from the mean OD for each sample (in coated wells). The mean OD for the negative control was also subtracted from the OD for the sample in the uncoated well. The OD for the uncoated well (minus the negative control) was then subtracted from the mean sample OD. This final OD value was used to calculate the amount of BoNT/C directly present in samples, using the standard BoNT/C curve and correcting for the original dilution factors. The amount of BoNT/C in samples, which had been enriched in CMC-1, was not quantified in this way due to amplification of toxin by the enrichment process. Samples were considered positive if the final OD value was equal to or greater than that for the 0.8ng/ml BoNT/C standard.

Specific antibody levels

IgG in serum

The negative control was automatically subtracted by the plate-reader software from both the OD readings for the coated and uncoated wells. The OD for the uncoated

well for each sample was then subtracted from the mean OD for that sample, in the coated well, to give the final OD value. The OD readings between plates were standardised by correcting the OD by reference to the positive control.

Serum was collected on more than one occasion from fourteen of the horses with CGS and six of the healthy controls. When more than one serum sample had been collected from a particular horse, the OD readings for the samples from each horse were averaged, to enable comparison of serum IgG levels between horses.

IgA in GI contents

Samples were determined as having detectable IgA to a particular antigen if the mean OD value obtained was greater than the mean + 3 x standard deviation (S.D.) of the negative control. This gives a probability of $p<0.0013$ for a negative sample falling outside this limit. The OD readings between plates were standardised by correcting the OD by reference to the positive control. When more than one faecal sample was obtained from an individual horse, the mean OD for the samples was calculated and used for the comparison of faecal IgA levels between horses.

IgG and IgA in colostrum and milk samples

Samples were determined as having detectable IgG or IgA to a particular antigen if the OD value obtained from the screening assays was greater than the mean of the negative control + 3 x S.D. of this control. The OD readings between plates were standardised by correcting the OD by reference to the positive control. Results for specific antibody levels were expressed both as corrected OD readings, and as OD readings per μg total IgA or per mg of total IgG.

Total antibody levels

The mean OD for the negative control was calculated and subtracted from the mean OD values for the standards and samples. The standard curve was then used to calculate the concentration of IgG or IgA in the samples, with correction of the results for the original dilution factors.

2.4.9 Statistical analysis

BoNT/C in GI samples

The levels of BoNT/C detected directly in the ileum and faecal samples were compared statistically between the categories of EGS disease and the controls by using the Mann-Whitney U test.

Specific antibody levels

OD values for IgG in serum, colostrum and milk samples and OD values for IgA in GI contents, colostrum and milk, were statistically compared between groups of horses by the Mann Whitney U test, using the SPSS package. An unpaired Students t-test (Sigmaplot) was also used to statistically compare the levels of IgG and IgA in colostrum and milk between different groups of horses. Linear regression analysis was carried out using Sigmaplot.

2.5 Isolation of lymphocytes from the lamina propria and Peyer's patches of equine gut associated lymphoid tissue (GALT).

2.5.1 Preparation of gut

A length of jejunum, proximal to the ileum, was collected at post-mortem. One end was tied off as tightly as possible, using nylon braided thread knotted three times with a surgical knot for the first knot. A section of gut approximately 20 cm in length was cut out (to include the knotted end). The serosal surface was washed with Hank's balanced salt solution without calcium and magnesium (GibcoBRL, Life Technologies Limited, Paisley, UK) containing 5mM HEPES (GibcoBRL) and 0.4g/l sodium bicarbonate (HBSS), pre-warmed to 37°C. The section of intestine was inverted by pushing the knotted end through with a glass rod. The section of intestine was then inflated by pouring HBSS (pre-warmed to 37°C) into it through a funnel. The HBSS was milked down by hand until all the folds of the mucosa were stretched out. The upper end of the sausage was clamped with bowel clamps and then tied off under pressure, to give a length of approximately 10cm of gut. The gut was examined for the presence of Peyer's patches. If no Peyer's patches were present, cells were isolated from the lamina propria; if Peyer's patches were present then cells from the Peyer's patches were isolated.

2.5.2 Removal of epithelium

The intestine was transferred to a wide necked autoclavable container with a lid that could be tightly sealed. Approximately 250ml of HBSS containing 1mM EDTA (pre-warmed to 37°C) was added, ensuring that there was enough solution to cover the gut, and shaken vigorously at 37°C for 20 min in an orbital incubator. The HBSS

was decanted and discarded, then another 250ml of HBSS containing 1mM EDTA (pre-warmed to 37°C) was added, and incubated for another 20 min at 37°C with vigorous shaking. This was repeated for at least six washes, or until the wash medium was clear i.e. all the epithelium was removed. For initial assays wash medium was retained from the final EDTA-wash and one of the earlier EDTA-washes for analysis of the cell content.

2.5.3 Isolation of cells from the lamina propria

The intestine was washed briefly with RPMI 1640 (Dutch modification; Gibco BRL) to remove as much wash HBSS/EDTA as possible. Approximately 250ml of RPMI 1640 containing 100units/ml collagenase (Sigma) was added and the intestine was incubated for 45 min at 37°C with vigorous shaking in the orbital incubator. The incubation medium was decanted and retained. A further 250ml of RPMI 1640 containing 100units/ml collagenase was added and incubated for 45 min at 37°C with vigorous shaking. The incubation medium from the first wash was centrifuged for 15 min at 300g, resuspended in HBSS without EDTA and centrifuged again for 15 min at 300g. The cells were resuspended in HBSS. This was repeated with the medium from the second digestion, and the cells from the first and second wash were combined. Cells were washed through a cell strainer (Falcon, Fred Baker Scientific, Runcorn, Cheshire, UK) to ensure there was a single cell suspension. Cells were resuspended in RPMI 1640 containing 10% FBS, streptomycin (100 μg/ml), and penicillin (100 units/ml) (10% complete RPMI).

2.5.4 Isolation of cells from Peyer's patches

The epithelium was removed from the gut as described above (Chapter 2.5.2). The Peyer's patches were then dissected out from the section of gut and peeled off from

the serosa. The Peyer's patch was then cut up into very small pieces and transferred to a conical flask. RPMI 1640 (50ml) containing 100units/ml collagenase was added and incubated for 45 min at 37°C on a magnetic stirrer. The incubation medium was decanted and retained, and a further 50ml RPMI 1640 containing 100 units/ml collagenase was added and incubated for another 45 min at 37°C with stirring. The incubation medium was decanted and retained; the remaining tissue from the digested Peyer's patch was put through a fine-meshed metal sieve and washed through with RPMI 1640 (without collagenase). The cells from each incubation were washed with HBSS (without EDTA) and pooled. Cells were washed through a cell strainer (Falcon) to ensure there was a single cell suspension. Cells were resuspended in 10% complete RPMI 1640.

2.5.5 Isolation of equine peripheral blood mononuclear cells (PBMCs)

Heparinised whole blood was diluted 1 in 4 in sterile PBS. PBMCs were separated by layering the diluted blood onto Histopaque 1077 (Sigma), and then centrifuging at 460g for 25 min. The interface layer containing the PBMCs was removed and washed twice in RPMI 1640; cells were centrifuged at 300g for 15 min. Cells were counted and resuspended in the appropriate final volume for the application.

2.6 Flow cytometry

2.6.1 Numbers of cells

Approximately 5 x 10^5 cells were added to each tube for flow cytometry. Ice-cold PBS (2ml) containing 0.01% BSA and 0.1% sodium azide (PBS/BSA/NaN_3) was added to each tube. Cells were centrifuged for 7 min at 300g and the supernatant was poured off, to remove the RPMI.

2.6.2 Blocking

Initial experiments were carried out to see whether a blocking step was necessary; cells were blocked with 0.2ml normal goat serum (NGS; Scottish Antibody Production Unit, Carluke, Lanarkshire, UK), diluted 1 in 200 in PBS/BSA/NaN_3, for 15 min at 4°C. Cells that were not blocked were resuspended in 0.2ml PBS/BSA/NaN_3. Cells then had 0.5ml of PBS/BSA/NaN_3 added and were centrifuged at 300g for 7 min. The supernatant was poured off.

2.6.3 Primary antibody

Three mouse monoclonal antibodies were used as primary antibodies: anti-human CD–18 (Dako, Glostrup, Denmark), anti-equine CD3 (J.L. Stott and M. Channel Blanchard, University of California, Davis, USA) and CVS 36 (M.A. Holmes, University of Cambridge, Cambridge, UK). The anti-equine CD3 monoclonal antibody recognises the equine equivalent of the CD3 antigen present on all T cells (Lunn et al, 1998). The CVS 36 monoclonal antibody is specific for all isotypes of equine immunoglobulin (Lunn et al, 1998), and therefore can bind to B cells through their expression of surface immunoglobulin (sIg). The anti-human CD18 antibody had been shown to cross-react with equine leucocytes, amongst other animal species (Jacobsen et al, 1993); CD18 is present on the surface of all leucocytes in conjunction with CD11. The negative control received no primary antibody, only PBS/BSA/NaN_3. Initial assays were carried out to determine the appropriate dilutions of anti-equine CD3, and CVS 36; 10µl of anti-CD18, diluted 1 in 10, was used in all the assays. Primary antibodies were diluted in PBS/BSA/NaN_3 and incubated for 30 min at 4°C. Cells were washed twice with 2 ml of PBS/BSA/NaN_3 and centrifuged at 300g for 7 min.

2.6.4 Conjugate

Anti-mouse IgG whole molecule–FITC conjugate $F(ab')_2$ fragment of sheep antibody (Sigma) was diluted 1 in 50 in PBS/BSA/NaN_3 and incubated at 4°C for 30 min. The cells were then washed twice with 2ml of PBS/BSA/NaN_3, as before. The cells were fixed by resuspension in 1ml of 0.1% formal saline and stored at 4°C.

2.6.5 Measurement of cells

Fluorescence was measured using a Coulter EPICS flow cytometer. Negative control gates were set at approximately 1% using the cells that had been incubated without first antibody.

2.7 ELISPOT assay for antibody secreting B cells

ELISPOT assays were developed to measure the number of total and antigen specific IgG and IgA secreting B cells in PBMCs and cells from GALT.

2.7.1 Coating and blocking of plates

Initial experiments were carried out to determine the optimum dilution for capture antibody for the assay to measure IgG-secreting cells. Multiscreen 96 well plates with individual mixed cellulose ester membranes (Millipore (UK) Ltd, Watford, UK) were coated with rabbit anti-equine IgG (H+L) antiserum (ICN) diluted in tris-buffered saline (TBS); 100µl was added to each well. Plates were incubated overnight at 4°C. Plates were washed three times with TBS containing 0.1% Tween 20 (TBS-T) (200µl/well); the third wash solution was left to sit in the wells for 5 min. The plates were then washed three times with TBS (200µl/well), and again the third

wash was left to sit in the wells for 5 min. Plates were then blocked with 10% complete RPMI 1640 (Gibco); 200µl were added to each well. Plates were incubated for 1-2 h at 37°C with 5% CO_2. The blocking solution was then thrown off.

For the ELISPOT to measure total IgA secreting cells, preliminary assays were carried out to determine the optimum dilution of capture antibody, anti-equine IgA mouse monclonal (BVS), as described above. For ELISPOT assays to determine antigen-specific antibody secreting cells, plates were coated with EDTA-extracted surface antigens of *C. novyi* type A and *C. botulinum* type C diluted in TBS at 30µg/ml, and BoNT/C diluted in TBS at 5µg/ml, as described above.

2.7.2 Addition of cells

Initial experiments were carried out to determine the optimum number of cells required for this assay. Cells were diluted in 10% complete RPMI 1640 and 100µl added to each well. Plates were then incubated for 4 h at 37°C with 5% CO_2. Plates were washed three times with TBS and then three times with TBS-T, with the third wash of each left to sit in the wells for 5 min; 200µl of wash buffer was added to each well.

2.7.3 Controls

Some wells were coated with 100µl BSA (0.4mg/ml) instead of capture antibody, to control for non-specific binding to the plate. Some wells received no cells, only 10% complete RPMI, as a negative control.

2.7.4 Primary detecting antibody

For ELISPOT assays to detect IgA secreting cells, rabbit anti-equine IgA (Fc) antiserum (Nordic Immunology) was diluted 1 in 400 in TBS-T containing 1% FBS and 100μl added to each well. Plates were incubated overnight at 4°C, and then washed as previously described.

2.7.5. Conjugate

For ELISPOT assays to detect IgG secreting cells, rabbit anti-horse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was diluted 1 in 10,000 in TBS-T containing 1% FBS, 100μl added to each well and the plates incubated overnight at 4°C. For ELISPOT assays to detect IgA secreting cells, goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma) was diluted 1 in 5,000 in TBS-T containing 1% FBS, 100μl added to each well and plates incubated for 90 min at 37°C. The plates were then washed as described previously with a final extra two washes with distilled water (200μl/well).

2.7.6 Substrate

BCIP/NBT (5-Bromo-4-chloro-3-9-indolyl phosphate and Nitro Blue Tetrazolium) liquid substrate solution (100μl; Sigma) was added to each well. The reaction was allowed to develop for 5 min before washing with distilled water to stop the reaction. The plate was then allowed to dry before spots were visualised and counted using a dissecting microscope with a vertical white light source at x15 and x30 magnification. The nitrocellulose discs from the bottom of the wells were later removed from microtitre plate using plate-sealing tape (Millipore) and spots visualised using x2.5 and x10 objectives with a light microscope.

2.8 Isolation and identification of Group III organisms

2.8.1 Isolation of Group III organisms

Ileum contents and faecal samples that had been inoculated into enrichment broth (CMC-1; Chapter 2.2.2) were plated out after 24 h of anaerobic incubation at 30°C onto pre-reduced Fastidious Anaerobe Agar (FAA; Oxoid Ltd, Basingstoke, Hampshire, UK.) containing 5% egg yolk emulsion (Oxoid) and 10μg/ml gentamicin (EYAg). Plates were incubated anaerobically at 30°C (anaerobic gas jar) or at 37°C (MK3 work station; Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) and checked at 24 h intervals for the presence of lipase and lecithinase positive colonies. Lipase-positive and lecithinase positive colonies were isolated by sub-culturing onto fresh EYAg plates, and finally into cooked meat broth to be kept as stock cultures.

2.8.2 Identification of group III organisms

Group III organisms were identified by their biochemical properties (lecithinase and lipase production), morphology when examined in wet films by phase contrast microscopy and Gram stain by light microscopy (Gram positive rods with subterminal oval spores), and gas liquid chromatography (GLC) profiles (Brown et al, 1996).

2.8.3 Bacterial strains

Bacterial type strains of Group III botulinum organisms - *C. botulinum* type C and D, and *C. novyi* type A - were obtained from the National Culture of Type Strains (NCTC; Central Public Health Laboratory, London, UK), Professor S. Nakamura (Kazazawa University, Japan), G.R. Smith (Institute of Zoology, Zoological Society

of London, UK), the Glaxo collection and the Microbial Pathogenicity Reasearch Laboratory (MPRL) collection (MPRL, Edinburgh University, Teviot Place, Edinburgh, UK) (Table 2.1).

Table 2.1: Group III type strains used

Lab ID	Organism	Original ID	Source
MPRL 3922	*C. botulinum* type C_{α}	NCTC 8264	NCTC
MPRL 2510	*C. botulinum* type C_{β}	NCTC 3732	NCTC
MPRL 3493	*C. botulinum* type C_{β}	NCTC 10914 (FH 6513)	Institute of Zoology
MPRL 4240	*C. botulinum* type C	KZ1577 (92-13)	Professor Nakamura
MPRL 3923	*C. botulinum* type D	NCTC 8265	NCTC
MPRL 2530	*C. novyi* type A	NCTC 538	NCTC
MPRL 2531	*C. novyi* type A	GR 2A	S277/64(B) Glaxo collection
MPRL 2532	*C. novyi* type A	NCTC 6737	NCTC
MPRL 2533	*C. novyi* type A	NCTC 6735	NCTC
MPRL 2534	*C. novyi* type A	2798	MPRL Original source unknown
MPRL 2535	*C. novyi* type A	4509	MPRL Original source unknown

2.8.4 Culture of strains for extraction of surface antigens

All media were pre-reduced before use, and incubation was carried out at 37°C in an anaerobic work-station (MK3, Don Whitley Scientific). Lyophilised cultures of type strains were inoculated into cooked meat broth and incubated for 24-48h to prepare stock cultures. Stock cultures of type strains and isolates were subcultured into 3 ml of protease peptone yeast extract broth containing 0.5% glucose (PPY-G; Brown et al, 1996), and after overnight incubation these starter cultures were used to inoculate 20 ml of the same medium. The 20 ml cultures were incubated overnight; the cells were then harvested.

2.8.5 EDTA extraction of surface antigens

Bacterial cells were harvested by centrifugation at 3,800g for 20 min. The pellet was resuspended in 10 ml of PBS pH7.3 (PBS tablets; Oxoid) and washed twice by centrifugation at 3,800g for 20 min. The bacterial cells were resuspended in 1ml PBS containing 10mM EDTA, incubated for 90 min at 45°C and then treated for 60 sec in an ultrasonic bath. The treated cells were transferred to microcentrifuge tubes and the cells removed by centrifugation at 12,700g for 2 min. The supernatants containing the surface extracted antigens were collected and centrifuged again at 12,700g for 2 min. The supernatants were collected and stored at –20°C before use.

2.8.6 Guanidine hydrochloride extraction of surface antigens

Bacteria were cultured, harvested and washed as for EDTA extraction. The pellets were then resuspended in 1.5 ml PBS containing 5M guanidine hydrochloride (GHCl), transferred to microcentrifuge tubes and incubated at room temperature for 2 h with shaking. Cells were then removed by centrifugation at 12,700g for 2 min;

the supernatants were collected and centrifuged again for 12,700g. The supernatants containing the extracted antigens were collected and the GHCl removed by overnight dialysis at 4°C against 6.25 mM Tris HCl, pH6.8 using a SpectralPor®Microdialyser (Orme Scientific Ltd., Middleton, Manchester, UK) with a membrane of 10,000 MW cut-off. The dialysed extracted antigens were stored at -20°C until required.

2.8.7 SDS-Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was done on 10% slab gels with the buffer system of Laemmli (1970) as described by Hancock and Poxton (1988). Samples (25-70μl) of EDTA and GHCl-extracted antigens from type strains and isolates were run so that there was approximately 50μg protein per track. Gels were stained with Coomassie blue as described by Hancock and Poxton (1988), or transferred to nitrocellulose for immunoblotting.

2.8.8 Immunoblotting with rabbit anti-*C. novyi* type A antiserum

The procedure is based on the method of Towbin et al (1979) as described by Hancock and Poxton (1988). After separation by SDS-PAGE, the surface antigens were transferred from the gel onto nitrocellulose paper (0.2μm pore size) overnight at 12V in electroblot buffer containing 0.025M Tris, 0.192M glycine and 20% (v/v) methanol at pH 8.3. The gel was then discarded and the nitrocellulose was washed in Tris buffered saline (TBS:0.02M Tris, 0.5M sodium chloride, pH7.5) for 10 min on shaker. All the following steps were carried out at room temperature with shaking. The nitrocelluose was blocked for 45 min with TBS containing 3% (w/v) gelatin, and then incubated for 3 h with *C. novyi* type A antiserum (raised against *C. novyi* type A MPRL 2530) diluted 1 in 200 in TBS containing 1% gelatin. The nitrocellulose was

then washed twice in TBS containing 0.025% Tween 20 (TTBS) for 10 min, and then incubated for 1 h with anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (Sigma) diluted 1 in 1000 in TBS containing 1% gelatin. The nitrocellulose was then washed twice in TTBS for 10 min, followed by three changes of tap water to remove all the Tween. HRP-colour development solution consisted of 60 mg 4-chloro-1-naphthol (BioRad Laboratories, CA 94547,USA) dissolved in 20ml of methanol, added, just before use, to 100ml TBS containing 60μl of a 30% solution of hydrogen peroxide. The HRP-colour development solution was added to the nitrocellulose and allowed to develop for 5-25 min. The reaction was stopped by rinsing several times with tap water and a final rinse with distilled water.

2.8.9 Immunoblotting with equine serum

The EDTA-extracted surface antigens of Group III type strains and isolates, separated by SDS-PAGE, were immunoblotted with equine serum as described above, except that equine serum was used at a dilution of 1 in 50. The conjugate, rabbit anti-horse IgG–HRP (Sigma), was used at a dilution of 1 in 1000.

2.8.10 Analysis of gels

The molecular weights of the bands were calculated using Phoretix 1-D gel analysis software (Phoretix International, Newcastle upon Tyne, UK) with Novex Mark12™ molecular weight standards (Novex™, San Diego, CA 92121, USA) for calibration of protein bands on Coomassie stained gels, and Novex See Blue™ pre-stained molecular weight standards for calibration of bands on immunoblots.

2.9 Polymerase chain reaction (PCR)

2.9.1 Extraction of DNA for PCR

Bacteria were cultured as described for extraction of surface antigens (Chapter 2.8.4). Bacteria were harvested by centrifugation at 11700g for 10 min at 4°C. The pellet was washed with 10 ml of 0.15M NaCl and 15mM sodium citrate pH7.0 (SSC) and centrifuged at 11700g for 10 min at 4°C. The pellet was resuspended in 1ml of 0.01M sodium phosphate buffer in 20% sucrose pH7.0 containing 2.5 mg/ml lysozyme (Sigma) and incubated at 37°C for 45 min. Tris-EDTA buffer pH7.4 (TE buffer; 10mM Tris and 1mM EDTA) containing 1% SDS was added (9 ml) with 1mg/ml proteinase K (Sigma) and incubated overnight at 37°C. An equal volume of phenol:chloroform mixture (10ml; Sigma) was added and shaken to form an emulsion before centrifugation at 4000g for 15 min at 4°C. The clear fraction at the top was removed using a glass pipette and transferred to a thick walled glass universal bottle; the phenol:chloroform separation was repeated until there was no white precipitate at the interface. The DNA was precipitated by the addition of 2.5 volumes of ice-cold ethanol and 0.1 volume of sodium acetate (3M). Nucleic acids were collected by centrifugation at 35000g for 30 min at 4°C; the supernatant was removed and the pellet allowed to dry before resuspension in 500µl TE buffer and storage at -20°C. DNA was visualised by separation on a 0.7% agarose gel and staining with ethidium bromide. The absorbance at A280/A260 was measured to quantify the amount of DNA present.

Initially, DNA was extracted from type strains as described above. However, for DNA extraction from isolates the process was scaled down, starting with a 1 ml volume of culture. A step to remove RNA was included, with incubation with 2µl

RNase (10μg/ml; Sigma) for 1 h at room temperature, after overnight incubation with proteinase K.

2.9.2 PCR reaction conditions

All PCR reactions were carried out in 100μl reaction buffer (GibcoBRL) containing 1.5mM $MgCl_2$, 100nM of each primer, 200μM of each dNTP, 5μl of DNA (extracted as described above) and 2.5 units Taq polymerase (GibcoBRL). The primers used were chosen from published sequences; the PCR cycling conditions for each primer set were as published for use with those primers. The primer sequences are given in Table 2.2, and cycling conditions in Table 2.3; BAC-1/BAC-2 primers were used at the same reaction conditions as the NOVA primers. The primers were used to amplify toxin genes from the DNA of type strains and isolates; DNA of type strains that were known to contain a particular gene were used as positive controls. A negative control, containing no template DNA, was also included in each PCR reaction.

2.9.3 Visualisation of PCR products

PCR products (10μl) were added to gel loading solution (2μl; Sigma) and run on 1% agarose gels in TAE buffer (40mM Tris and 2mM disodium EDTA, pH8.0) containing ethidium bromide (0.1mg/ml). Products were visualised under ultraviolet light. Initially, a ϕX174 Hae III digest (Sigma) was used as a molecular weight marker to size products; the digest had fragment sizes between 1,353 and 72 base pairs. Later the Readyload™ 100bp ladder (GibcoBRL) was used to size products; this DNA ladder gives bands between 100bp and 2000bp.

Table 2.2: Target genes for amplification by primer pairs and primer sequences.

Target gene	Primer name	Primer sequence	Product size	Reference
BoNT/C	CS11 CS22	5'-ATACACTAGCTAATGAGCCTG-3' 5'-TGGAGTATTGTTATTCCCAGG-3'	290 bp	Takeshi et al, 1996
BoNT/D	DS11 DS22	5'-GTGATCCTGTTAATGACAATG-3' 5'-TCCTTGCAATGTAAGGGATGC-3'	497 bp	Takeshi et al, 1996
C2 component I	C2CI-F C2CI-R	5'-AAGGAAGATAAAACAAAAAT-3' 5'-CCTAATGATACAAATGAAAA-3'	310 bp	Fujii et al, 1996
C2 component II	C2CII-F C2CII-R	5'-ATTATTCTCACACATGGCAAAATACAAG-3' 5'-GATTTACTGCCATTATAATAAAAGT-3'	1093 bp	Kimura et al, 1998
***C. novyi* alpha toxin**	NOVA-F NOVA-R	5'-GGTGCGATTCAAGAGGCCACA-3' 5'-CGCTCCTAGCAGTCCCGAAAT-3'	260 bp	Hofmann et al, 1995
16s rRNA (Universal)	BAC-1 BAC-2	5'-ACGGCGCAGACTCCTACGGCAGGC-3' 5'-GGGTTGCGCTCGTTGCGGCACTTA-3'	763 bp	Franciosa et al, 1994

Table 2.3: Cycling conditions for PCR reactions with the different primer pairs.

Primer pair	Initial denaturation	Cycling reaction		Final extension
		No. of cycles	Conditions	
CS11/CS22	10 min at 95°C	25	1 min at 94°C 1 min at 55°C 1 min at 72°C	3 min at 72°C
DS11/DS22	10 min at 95°C	25	1 min at 94°C 1 min at 55°C 1 min at 72°C	3 min at 72°C
C2CI-F/C2CI-R	3 min at 94°C	35	45 s at 94°C 2 min at 55°C 1 min at 72°C	5 min at 72°C
C2CII-F/C2CII-R	3 min at 94° C	40	45s at 94°C 1 min at 53°C 3 min at 72°C	5 min at 72°C
NOVA-F/NOVA-R	3 min at 94°C	30	1 min at 95°C 1 min at 48°C 1 min at 72°C	5 min at 72°C

Chapter Three

Detection of BoNT/C in the GI tract

3.1 Results

3.1.1 Direct Detection of BoNT/C in GI contents

The presence of BoNT/C, in the ileum contents and faeces of horses with and without grass sickness, was investigated using a sandwich ELISA specific for BoNT/C. Overall, BoNT/C was detected directly in 45% (13/29 horses) of samples of ileum contents from horses with grass sickness compared with only 4% (1/28 horses) of controls. BoNT/C was directly detected in 44% (20/45 horses) of faecal samples from horses with grass sickness compared with only 4% (3/77 horses) of controls.

BoNT/C was directly detected in the ileum contents of a greater proportion of chronic (50%) and acute cases (50%) than subacute cases (29%) (Fig. 3.1). The toxin was also detected in the faeces of a greater proportion of chronic (50%) and acute cases (46%) than subacute cases (17%) (Fig. 3.1).

The one control case with detectable toxin in the ileum had been initially clinically diagnosed as having acute grass sickness. However, histopathology at post-mortem failed to show the characteristic lesions, and the final diagnosis was uncertain. The three control horses that had detectable toxin in their faecal samples, included two orthopaedic cases (one of which had had chronic grass sickness sixteen months previously) and a horse with surgical colic.

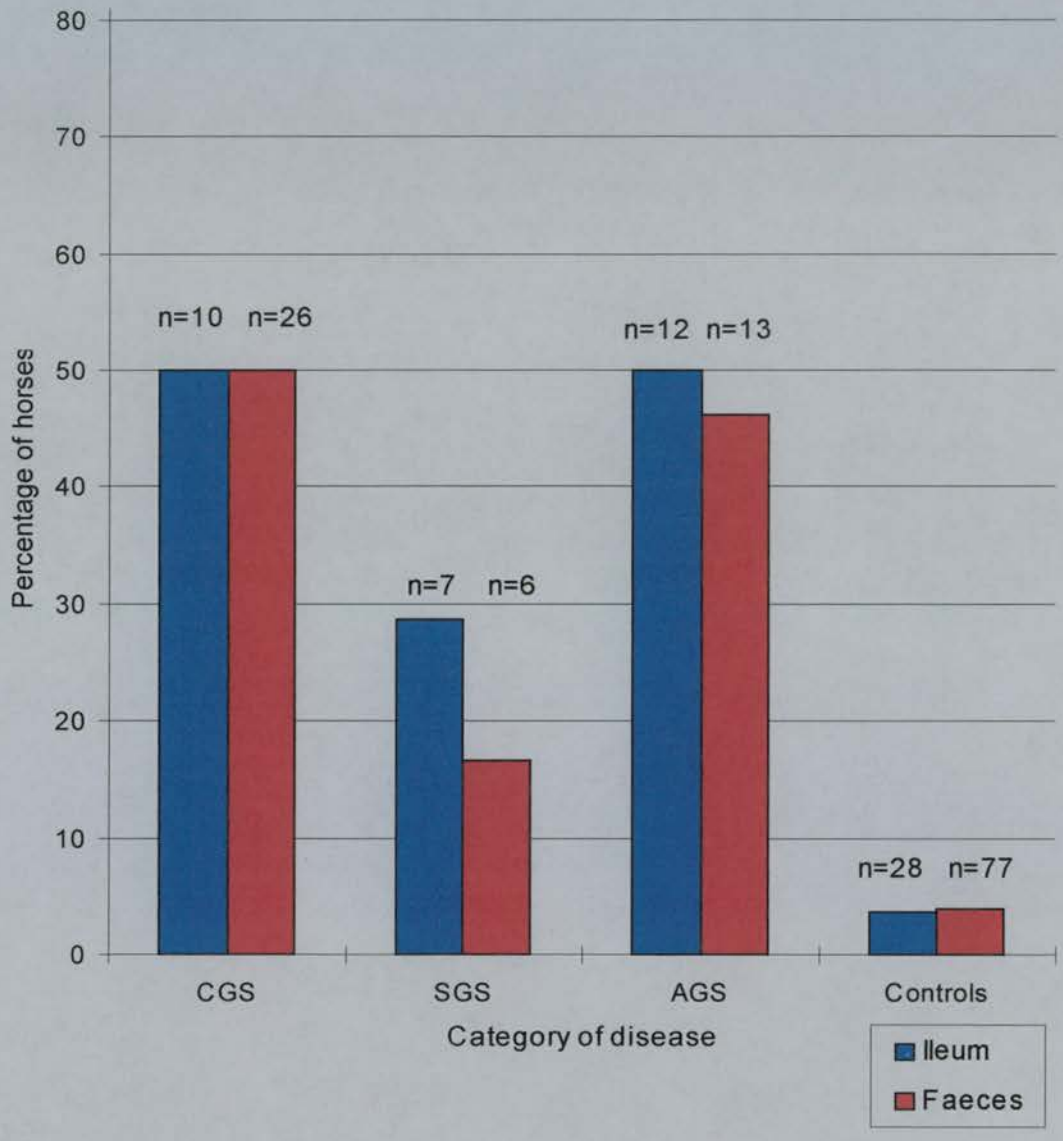

Figure 3.1: Percentage of horses with directly detectable BoNT/C in the ileum and faeces, i.e. before enrichment. The number on top of each bar represents the number of horses in each category.

3.1.2 Quantitation of BoNT/C in GI contents

The amount of BoNT/C detected in the ileum and faeces was quantified as ng of BoNT/C per gram of wet weight sample, using a standard curve (Fig. 3.2). Comparisons between samples should be made with caution as the concentration of toxin is calculated using the wet weight of the sample, and there will be some variation in consistency between samples. The volume of gut contents can vary significantly between horses, which may also affect toxin detectability.

There is a statistically significant difference in the levels of toxin detected in the ileum, between the CGS group and controls ($p=0.001$), between the SGS group and controls ($p<0.05$) and between the AGS group and controls ($p<0.001$) (Fig. 3.3a). For the faecal samples, there is a statistically significant difference in the levels of toxin directly detected between the CGS group and controls ($p<0.001$), and between the AGS group and controls ($p<0.001$) (Fig. 3.3b).

The highest level of BoNT/C (10430ng/g) was detected in the faeces of a horse with chronic grass sickness of 15 days duration that was sampled at post-mortem. The ileum of this horse also contained detectable toxin (79.2ng/g). The one control horse in which BoNT/C was detected in the ileum had a low level of toxin (10ng/g wet weight sample), but note that this horse had symptoms typical of grass sickness.

3.1.3 Detection of BoNT/C in GI contents after enrichment

In order to demonstrate the presence of a BoNT/C-producing organism in the GI tract, samples of GI contents were enriched in culture medium and the culture supernatant examined for the presence of BoNT/C, by ELISA. After enrichment in

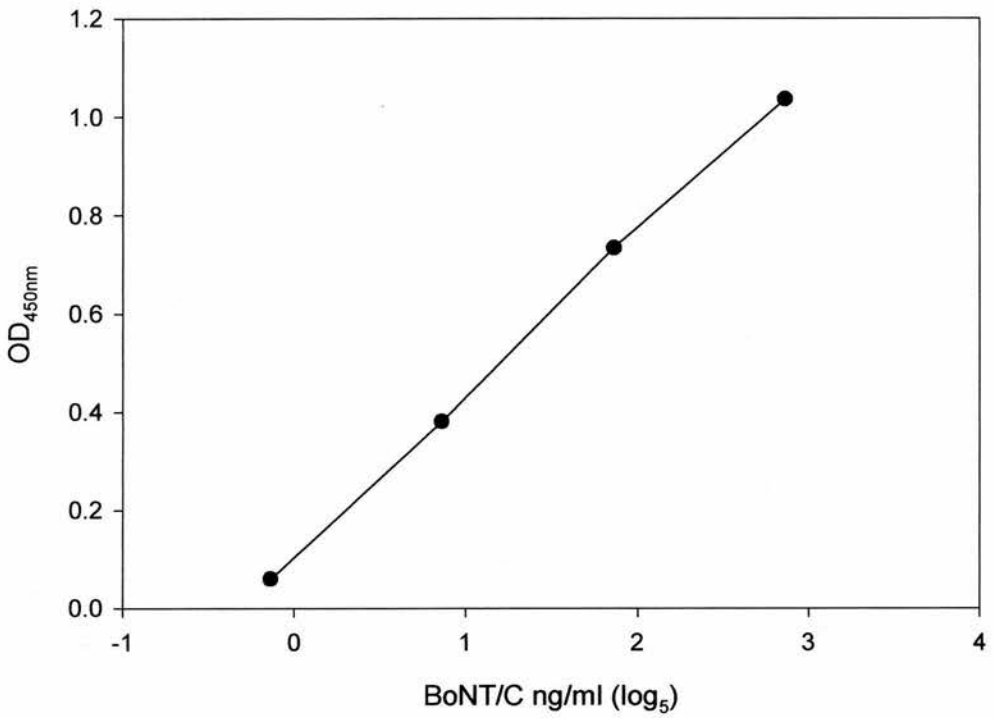

Figure 3.2: Example of BoNT/C standard curve with BoNT/C diluted fivefold between 100ng/ml and 0.8ng/ml

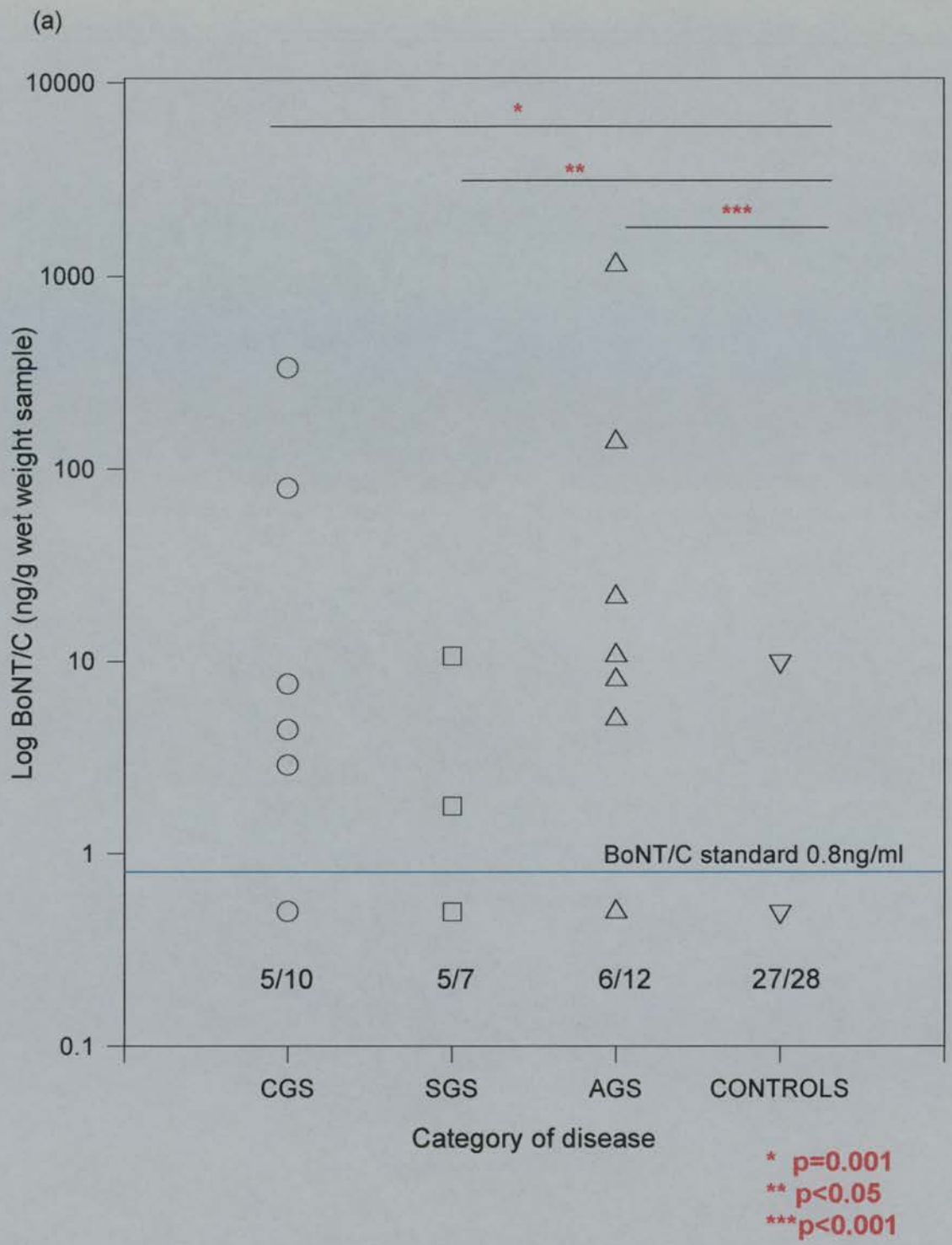

Figure 3.3: Scatterplot showing the range of BoNT/C concentrations directly detected in (a) ileum samples and (b) faecal samples. The number of samples in each category which did not have detectable toxin are represented as a proportion of total sample number in each group, by the numbers below the reference line of the BoNT/C standard of 0.8ng/ml, on the graph. The detectable limit of the assay is considered to be equivalent to 0.8ng/ml of the BoNT/C on the standard curve from the ELISA. Statistical differences between groups are shown. The mean amount of BoNT/C in the ileum was 42.9ng/g wet weight sample for horses with CGS; 1.8ng/g for SGS; 110.5ng/g for AGS and 0.4ng/g for controls. The mean amount of BoNT/C in the faeces was 230.5ng/g wet weight sample for horses with CGS; 3.8ng/g for SGS; 504.9ng/g for AGS and 0.3ng/g for controls.

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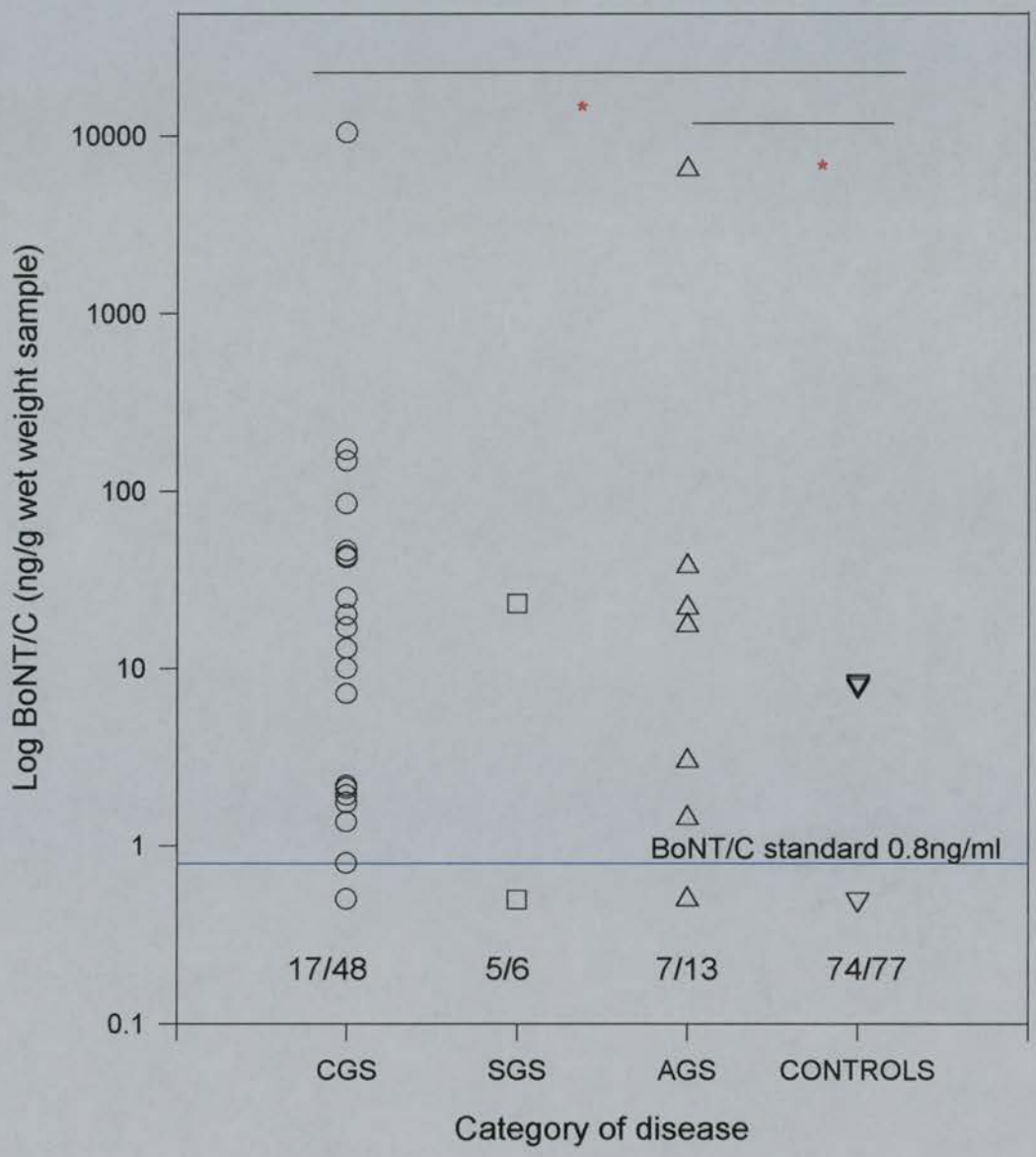
(b)


Log BoNT/C (ng/g wet weight sample)
10000
1000
100
10
1
0.1
BoNT/C standard 0.8ng/ml
17/48
5/6
7/13
74/77
CGS
SGS
AGS
CONTROLS
Category of disease

* p<0.001

CMC-1 medium, BoNT/C was detected in 48% (14/29) of ileum samples and 44% (20/45) of faecal samples from horses with grass sickness, compared to only 7% (2/27) of ileum samples and 8% (6/72) of faecal samples from controls. Not all samples that had detectable levels of toxin directly were positive for BoNT/C after enrichment, and vice versa.

The two control horses, out of 27, with detectable BoNT/C in the ileum after enrichment were the horse clinically diagnosed as AGS but not histopathologically confirmed, and a horse with a hepatic disorder. The six horses, of 72 controls, with detectable BoNT/C in the faeces after enrichment were two orthopaedic cases (one of which had had grass sickness 16 months previously), two healthy horses, a horse in-contact with a case of grass sickness, and a horse with ileus.

Ileum samples from horses with acute grass sickness had a higher proportion of samples positive for BoNT/C (75%), after enrichment, than SGS (43%) and CGS (20%) (Fig. 3.4). Faecal samples from horses with AGS also had a higher proportion of samples positive for BoNT/C (54%), after enrichment, than CGS (42%) and SGS (33%) (Fig. 3.4).

3.1.4 Longitudinal study of horses with CGS

Twelve chronic cases had more than one faecal sample collected, over the course of the disease. Eleven of these 12 horses were negative for BoNT/C when initially sampled; five of these 11 had directly detectable levels of BoNT/C in later faecal samples (whilst the other six remained negative). One horse had detectable BoNT/C in both faecal samples that were collected. Table 3.1 shows how the levels of detectable toxin in the faeces of the six horses varied over the course

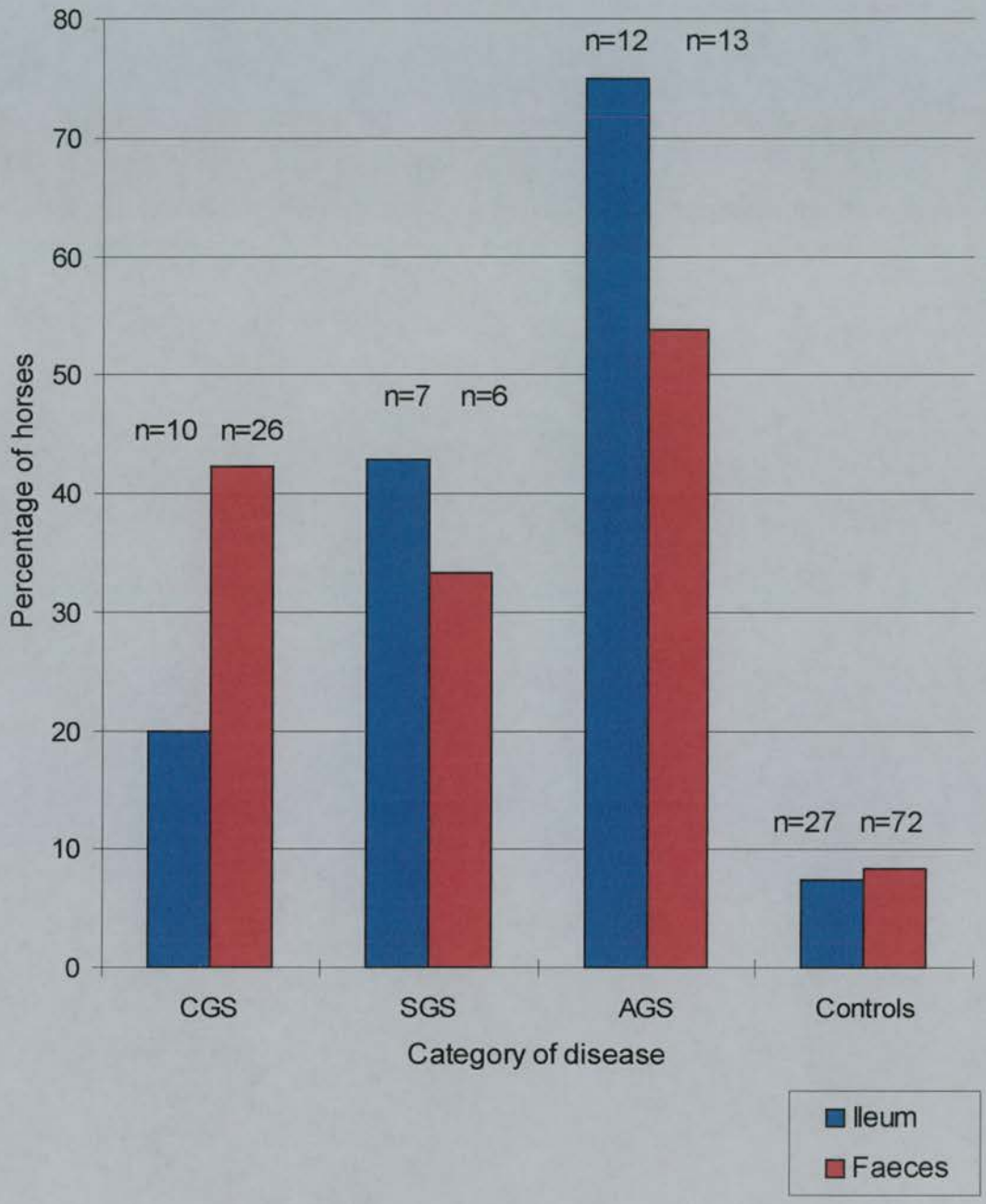


Figure 3.4: Percentage of horses with detectable BoNT/C in the ileum and faeces after enrichment. The number on top of each bar represents the number of horses in each category.

of the disease. Five of the six horses recovered; the sixth was euthanased, and a level of 335ng/g BoNT/C was detected in the ileum at post-mortem. Of the six horses that had no detectable toxin, three recovered and three were euthanased (one of these had toxin detectable after enrichment).

Table 3.1: Detection and quantitation of BoNT/C, before and after enrichment, in multiple faecal samples taken from horses with chronic grass sickness.

Horse reference number	Duration of disease (days)	Day sampled	BoNT/C detected before enrichment (ng/g wet wt sample)	BoNT/C after enrichment	Outcome
97/150	75	5	No	No	Euthanasia
		75	7.2	Yes	
98/540	47	38	No	No	Recovered
		47	42	Yes	
97/545	40	6	No	Yes	Recovered
		39	1.36	No	
97/623	67	23	147.5	No	Recovered
		66	43	Yes	
97/834	54	1	No	No	Recovered
		22	No	No	
		39	0.7	No	
		43	1.75	No	
97/987	47	10	No	No	Recovered
		14	2.1	No	
		21	171	Yes	
		28	25	Yes	
		35	17	No	
		41	10	No	

3.1.5 Detection of BoNT/C in GI contents by direct detection and/or enrichment.

BoNT/C was detected, both before and after enrichment, in the faeces of 27% of horses with CGS, 17% of horses with SGS and 46% of horses with AGS, compared to 3% of controls. BoNT/C was detected, both before and after enrichment in the ileum contents of 20% of horses with CGS, 14% of horses with SGS and 42% of horses with AGS, compared to 4% of controls.

When the detection methods are combined for individual horses i.e. when toxin can be detected in the ileum and/or faeces by direct detection and/or enrichment, then 74% of acute cases, 67% of subacute cases and 67% of chronic cases have detectable toxin compared to 10% of controls.

3.1.6 Detection of BoNT/C in other animals with dysautonomia

The intestinal contents of cats, rabbits and a hare, all with histologically confirmed dysautonomia, were investigated for the presence of BoNT/C and BoNT/C-producing organisms. BoNT/C was directly detected in the intestinal contents of 69% (11/16) of cats with dysautonomia but not in the GI contents of the two cats that but did not have dysautonomia (these two cats had diarrhoea and were part of the same breeding colony as 13 of the cats with dysautonomia). The levels of BoNT/C present in the intestinal contents ranged from 5.3 to 1198.8ng BoNT/C per g wet weight sample. After enrichment, BoNT/C was detected in 56% (9/16) of cats with dysautonomia and in one of the cats with diarrhoea. BoNT/C was detected in the intestinal contents either directly and/or after enrichment in 81% (13/16) of the cats with dysautonomia. Intestinal contents from only one hare and two rabbits with dysautonomia were investigated: BoNT/C was detected directly in the intestinal

contents of the hare and one of the rabbits. Levels of 74 ng BoNT/C per g of wet weight sample were detected in the hare and 2375 ng of BoNT/C per g of wet weight sample in the rabbit. After enrichment, BoNT/C was detected only in the sample from the rabbit that had also had BoNT/C detectable directly.

3.2 Discussion

3.2.1 Detection of BoNT/C in horses

The detection of BoNT/C, before and after enrichment, in horses with grass sickness shows that there is a strong association between the presence of BoNT/C and BoNT/C-producing organisms, in the equine GI tract, and a clinical diagnosis of equine grass sickness. Toxin was detected antemortem in faecal samples from chronic cases and post-mortem in ileum and faecal samples of all categories of grass sickness. Toxin, however, was not detected in all the samples from grass sickness cases. This may be a reflection on the sensitivity of the assay; the ELISA is approximately 100 times less sensitive than the conventional mouse (lethality) bioassay that can be used for toxin testing. Horses are extremely sensitive to botulinum toxins, and the level of circulating toxin in horses with classical botulism is usually below the threshold for detection by the mouse bioassay (Whitlock and Buckley, 1997). Only a very small amount of toxin may be needed to exert a clinical effect. The absence of detectable toxin in the ileum and faeces may also be due to the rapid absorption of BoNT/C from the gut, destruction by proteases or neutralisation of the toxin by mucosal antibodies. Specific IgA to BoNT/C has been detected in the ileum contents and faeces of horses with grass sickness (Chapter 6.1.2).

Another factor that may affect the ability to detect BoNT/C is the point in the disease at which the sample was taken (as well as the volume of gut contents present at time of sampling). This is of particular relevance with respect to the chronic cases, where the disease can last from several weeks to several months. The sampling of horses with chronic grass sickness over the course of the disease showed that the toxin could be detected in the faeces of six horses towards the end of the disease, whereas five of these horses had been negative when tested earlier. Five of these six horses were considered to have recovered but still had toxin in their faeces close to or on the date that they were sent back to their owners. The ability to detect toxin in the faeces of these horses is therefore not necessarily an indicator for a poor prognosis. Horses may be able to tolerate a sub-clinical level of toxin in the faeces: toxin produced in the large intestine may be poorly absorbed, or destroyed, in healthy horses. Colonisation of infant mice with *C. botulinum* does not cause illness despite the detection of up to 2000 adult mouse intraperitoneal 50% lethal doses of toxin in the colon and caecum. This is thought to be due to localisation of the toxin to the colon where it would be poorly absorbed (Sugiyama, 1980).

The presence of toxin in the ileum is probably a more significant finding with respect to clinical outcome. BoNT/C was detected, pre-enrichment, in the ileum contents of only one of 28 controls. As noted previously, this horse had been clinically diagnosed as an acute grass sickness case, however the diagnosis was not confirmed histologically at post-mortem. This case highlights the need to investigate the possible role of *C. botulinum* type C in cases presenting with clinical symptoms similar to grass sickness. The ileum sample from this horse, as well as an ileum sample from a horse with a hepatic disorder, had toxin present after enrichment, demonstrating the presence of *C. botulinum* type C in the upper GI tract of only two control horses.

It is interesting to note that the acute grass sickness cases had a higher mean level of toxin in the ileum and faeces, than the other categories. It is possible that acute grass sickness may result from exposure to a large amount of toxin whereas the chronic form may result from the exposure to smaller amounts of toxin over a longer period of time (Doxey et al, 1995a). The clinical severity and duration of the disease correlates to the extent of enteric neuronal damage. Enteric neuropathy is widespread and more severe in acute cases but in chronic cases the neuronal loss is less and tends to be localised to the distal small intestine (Scholes et al, 1993a). A higher percentage of horses with acute grass sickness had detectable BoNT/C–producing organisms in both the ileum and faeces compared to horses with chronic or subacute grass sickness; this may be evidence of a wider distribution of *C. botulinum* type C in horses with acute grass sickness, which may account for the more widespread enteric neuronal damage observed in these horses.

The hypothesis that is being investigated is that equine grass sickness is caused by a toxicoinfection with *C. botulinum* type C. The diagnosis of a toxicoinfection requires the demonstration of the presence of both toxin and toxin-producing organism in the affected animal, as is the case for infant botulism (Hatheway, 1979). 42% of horses with acute grass sickness were found to have both BoNT/C and a BoNT/C-producing organism in the GI tract, supporting the hypothesis of a toxicoinfection. The presence of *C. botulinum* type C in the samples is demonstrated by detecting the toxin in enrichment culture supernatants. The production of this neurotoxin is encoded on a prophage that can be lost or acquired during growth and sporulation. Therefore the detection of BoNT/C after enrichment does not necessarily mean that an organism was producing toxin in vivo, and conversely an organism that was producing toxin in vivo may not be detected in culture due to loss of the prophage. *C. botulinum* type C is a particularly fastidious

organism, and successful culture and isolation can be very difficult. A significant number of samples do demonstrate the presence of *C. botulinum* type C, for example 75% of ileum samples from acute cases. The samples, which do not have detectable toxin after enrichment, could be due to the absence of organism in the enriched sample, prophage instability, or inhibition of growth or toxin production by other organisms present. *Bacillus* and other clostridial species have been shown to be inhibitory to the growth of *C. botulinum* type C (Sandler et al, 1998; Graham 1978). The optimum growth temperature for *C. botulinum* type C appears to vary for different strains between 30-42°C, and the amount of toxin produced in the culture supernatants has been shown to be inversely related to growth (Hyun and Sakaguchi, 1988). This appears to be due to the intracellular accumulation of toxin.

BoNT/C has been shown to bind to specific polysaccharide moieties of the peptidoglycan of *C. botulinum* type C strains. Bacterial cell-bound toxin has increased oral toxicity compared to free toxin when given to chickens (Hyun and Sakaguchi, 1989). It has been hypothesised that cell bound toxin may be important in the pathogenesis of toxicoinfectious botulism in broiler chickens. The toxin is bound to the cell surface via the non-toxic component with which the toxin is associated; binding occurs at pH2 (Hyun and Sakaguchi, 1989). The ingestion of *C. botulinum* type C with intracellular toxin was also more toxic to chickens than the cell-free toxic supernatant (Hyun and Sakaguchi, 1989). Intracellular and cell-associated toxin may play a role in the toxicoinfection in other animals, including grass sickness.

Only six of 72 control faecal samples contained an organism capable of producing BoNT/C after enrichment. This may indicate the low carriage of toxigenic organisms

in the normal equine population, or, it may suggest that there are factors preventing detection of the organisms. Characterisation of isolates from horses with grass sickness has shown that they resemble *C. novyi* type A, rather than *C. botulinum* type C (Chapter 4). Whilst this does not preclude their ability to produce BoNT/C if infected with the appropriate bacteriophage, it is possible that non-toxigenic organisms are normally carried in the GI tract. These non-toxigenic organisms would not be detected by the enrichment method used in this study. A PCR for the detection of *C. botulinum* type C and related organisms is currently being developed to enable the more accurate assessment of the carriage of these organisms in the equine population.

There are some differences in opinion as to the clinical relevance of *Clostridium botulinum* isolated from the GI tract of animals (Kinde et al, 1991). The organism is commonly found in soil samples and aquatic sediments (Hatheway, 1990) but its distribution can vary considerably within the local area (Smith, 1975b). Different types of *C. botulinum* are found in different geographical areas; for example *C. botulinum* type B is predominant in the soils in the eastern US, and consequently this serotype is the major cause of adult equine botulism and shaker foal syndrome in that area. Type C is a cause of equine botulism in Europe, but is less common in the US despite being the predominant type isolated form soils in Florida (Johnston and Whitlock, 1987). Types C and D have been described as obligate parasites of animals and birds, seldom being isolated from the soil unless from an area where birds or domestic animals congregate (Smith, 1975b). Spores of *C. botulinum* are commonly found in the faeces of herbivores without clinical symptoms (Kinde et al, 1991). However, it has been reported that spores are rarely isolated from normal, healthy horses that are not at risk from botulism and that toxin has not been isolated from the faeces of an adult animal antemortem (Johnston and Whitlock, 1987).

The results of this investigation have demonstrated that both the organism and the toxin can be detected in the faeces of horses antemortem, as well as the ileum of horses post-mortem; these animals did not present with classical botulism but equine grass sickness. It is important to address the question why these horses do not present with classical symptoms of botulism. Equine grass sickness does share some clinical similarities to botulism, particularly in the acute form (Tocher et al, 1923, Greig, 1928). However, many of the neuromuscular symptoms characteristic of classical botulism are not observed. The chronic and subacute forms develop a very tucked up 'greyhound-like' abdomen. This is strikingly similar to the 'wasp-waist' seen when laboratory mice are inoculated with botulinum toxins (I.R. Poxton, personal communication).

Equine grass sickness is thought to be associated with intestinal colonisation of *C. botulinum* with in-vivo production of toxin(s), rather than ingestion of pre-formed toxin - which is the cause of classical botulism. Consequently the enteric nervous system of the horse is exposed to a high dose of locally produced toxin, causing severe neuronal damage and initiating the GI dysfunction associated with the disease. It is proposed that there is a concentration gradient affect away from the focus of toxin production, perhaps generated by axonal transport, with the more peripheral ganglia being affected to varying degrees. If this is the case, then it is possible that insufficient neurotoxin reaches the peripheral cholinergic synapses at the neuromuscular junction to cause the neuromuscular symptoms observed in botulism (in classical botulism the pre-formed toxin is ingested and rapidly absorbed, becoming systemic). Hodson et al (1984b) observed that changes in the coeliaco-mesenteric ganglion resembled those of classical retrograde degeneration after axotomy. This supports the hypothesis that the primary lesion is in the GI tract.

There are some differences in the clinical presentation of toxicoinfectious botulism in humans (infant botulism, and the extremely rare adult form) compared to ingestion of pre-formed toxin, particularly with respect to GI symptoms. Nausea and vomiting occur in food-borne botulism but not in infant botulism; constipation is a prominent feature of infant botulism. Disruption or immaturity of normal flora, disturbance in gut motility, and compromisation of the gastric acid barrier, can all predispose to intestinal infection with *C. botulinum* in humans (Chia et al, 1986). Signs and symptoms of autonomic dysfunction, both parasympathetic and sympathetic, may be prominent in human botulism (Tacket and Rogawski, 1989); the disease has also been observed in humans where there is mainly cholinergic autonomic dysfunction with no neuromuscular involvement (Jenzer et al, 1975).

The symptoms of equine grass sickness may result from the involvement of the three toxins produced by *C. botulinum* type C. All three toxins could in theory cause the neuronal damage observed in EGS. To date only the presence of the C1 toxin (BoNT/C) has been investigated; reagents to detect the C2 and C3 toxins were not obtainable. However, the C2 and C3 toxins, together with the in-vivo growth of the organism may be responsible for the difference in clinical symptoms between grass sickness and botulism. C2 toxin has been shown to cause hypotension, haemorrhage, cytotoxicity and increase in vascular permeability (Aktories et al, 1986). In the intestine it has been shown to cause intestinal secretion, fluid accumulation, histopathological changes (Ohishi, 1983b; Ohishi and Odagiri, 1984) and inhibition of contraction of guinea pig ileum myenteric plexus longitudinal muscle preparation (Mauss et al, 1989). However, the role of the C2 toxin in the pathogenesis of disease in vivo has yet to be determined.

Ultrastructural investigation of the neuronal lesions of grass sickness has indicated marked abnormalities of the neuronal cytoskeletal, cytoplasmic and secretory proteins with disruption or loss of the Golgi associated membranes (Griffiths et al, 1993). It is possible that the C2 and C3 toxins could cause the ultrastructural damage observed. Actin-polymerisation has a role in governing secretion from neural cells and consequently is thought to play some role in neurotransmission (Viviani et al, 1996). All three of the toxins produced by *C. botulinum* type C could therefore affect neurotransmission in some way, either by their effect on the cytoskeleton or the secretory vesicles of the exocytic pathway. The neurotoxicity of BoNT/C (Williamson and Neale, 1998) may be able to account for some of the neuronal degeneration that is observed in grass sickness. The type C neurotoxin is the only botulinum neurotoxin that has been shown to cause overt neuronal degeneration in vitro; the other botulinum neurotoxins have only a functional effect.

The enteric cholinergic neurones from the ileum of horses with acute grass sickness exhibit altered cholinergic mechanisms, with a reduction in the release of acetylcholine (Murray et al, 1994). Botulinum neurotoxins inhibit the release of acetylcholine from cholinergic nerves. The prokinetic drug Cisapride, an indirect cholinergic agent, facilitates the release of acetylcholine from the postganglionic nerves of the myenteric plexus in the gut. This drug has been shown to be of some therapeutic benefit in the treatment of selected cases of chronic grass sickness (Milne et al, 1996). Acetylcholine release is stimulated from the remaining morphologically normal neurones and increases gut motility.

The hypothesis that grass sickness is caused by toxicoinfection with *C. botulinum* is, to some extent, a reinvestigation of the hypothesis originally put forward by Tocher et al in 1923. Their work was not widely accepted at the time. One of the major

criticisms made, related to their inability to prove conclusively that the organism they had isolated from grass sickness horses was *Bacillus botulinus* - they could only say that it morphologically and toxigenically resembled this organism. Furthermore there was a lack of acceptance, by Tocher's contemporaries, of the ability of *C. botulinum* to produce toxin within a living animal (Anon, 1927; Greig, 1942). Serotype C of *C. botulinum* was only first identified in 1922 (Bengston, 1922; Seddon, 1922). Therefore, this was almost certainly not the serotype that was vaccinated against in 1922, particularly as the antitoxin used was of US human origin. *C. botulinum* type C had not been identified as a cause of human botulism at that point, and there have only been five suspected human type C cases since (Sonnabend et al, 1985). *C. botulinum* type A and B neurotoxins, responsible for causing botulism in humans, are not immunologically cross-reactive with BoNT/C.

At this point, it is not possible to say whether *C. botulinum* type C is the primary cause of grass sickness, a causal co-factor, or whether the organism grows and produces its toxins as a secondary event due to reduced gut motility. Investigation of horses with GI stasis for reasons other than grass sickness could assist in determining whether type C toxin is produced as a consequence of this stasis. Only five faecal samples and two ileum samples from horses with GI dysfunction, but not grass sickness, have been assayed to date. This has shown the presence of toxin in one faecal sample from a horse with colic, and in one ileum sample from a horse that had presented clinically with acute grass sickness, but the diagnosis was unknown after post-mortem. The number of samples investigated is not sufficient to determine whether toxin is produced after the onset of GI stasis, independently of grass sickness.

3.2.2 Detection of BoNT/C in other species with dysautonomia

The clinical, pathological and epidemiological similarities seen between dysautonomias in cats, rabbits and hares, and equine grass sickness have led to the suggestion of a common aetiology (Pollin and Griffiths, 1992). The detection of BoNT/C, before and after enrichment, in the intestinal contents of a rabbit, hare and cats with dysautonomia, supports both the theory of a common aetiological agent and the role of toxicoinfection with *C. botulinum* type C in the cause of equine grass sickness.

A high percentage of cats with dysautonomia had BoNT/C present in the intestine and/or a BoNT/C-producing organism. The demonstration of *C. botulinum* type C in cats with dysautonomia is perhaps the most surprising as the diet and intestinal physiology of cats and horses are quite different; rabbits and hares are herbivores and exposed to the same grazing environment as horses. It would therefore be likely that hares and rabbits could also acquire or carry similar enteric organisms as the horse. The cats in question were part of a closed colony in a laboratory. Either *C. botulinum* type C is carried by cats or was introduced by an external source, possibly in the food.

3.2.3 Summary

In summary, there is evidence to support the hypothesis that equine grass sickness (and possibly other similar dysautonomias) is a toxicoinfection with *C. botulinum* type C, with toxin production and absorption predominantly in the ileum. The organism is either normally present in the large intestine, and overgrows into the ileum, or the organism is taken up in spore form and is able to germinate in the ileum, following a change in the environment of the GI tract due to an environmental

trigger. Horses are notoriously sensitive to changes in diet, and grass sickness is associated with grazing, and a change in pasture. The trigger therefore could be a nutritional one, affecting the gut flora, and facilitating the colonisation of the intestine with *C. botulinum* and its subsequent toxin production. The disease severity is likely to be influenced by an interaction of factors such as the amount of toxin the horse is exposed to, the local mucosal and systemic immune response to the toxin, the immune status at the onset of disease and the inherent susceptibility of the horse.

Whether *C. botulinum* type C is the cause of grass sickness or whether it is involved as a secondary event, the detection of high levels of toxin in the ileum at post-mortem, and ante-mortem in faeces in horses with equine grass sickness, together with the known sensitivity of horses to botulinum toxins, supports a significant role for the involvement of *C. botulinum* type C in equine grass sickness.

Chapter Four

Isolation and characterisation of Group III organisms

4.1 Results

4.1.1 Isolation of Group III organisms

Sixteen isolates, identified as Group III organisms, were isolated from enrichment cultures of GI contents. Group III organisms include *C. botulinum* type C and D and *C. novyi* type A. Twelve Group III organisms were isolated from the GI contents of nine horses; three Group III organisms were isolated from the small intestine of a hare and one from the small intestine of a cat (Table 4.1). All of these organisms were isolated from animals that had dysautonomia at the time of sampling, or had previously had dysautonomia and recovered, or had recently been in contact with a horse with dysautonomia (Table 4.1). Repeated subculturing onto fresh EYA plates was required before a pure culture could be obtained of any of these isolates, due to overgrowth on the plates by other bacteria present in the GI samples.

The Group III isolates, from these animals, produced both lipase and lecithinase and were Gram positive (or Gram variable) rods with subterminal spores. The isolates all produced proprionic and butyric volatile fatty acids (VFA) and varying amounts of acetic and valeric VFA (Table 4.2). These GLC profiles are characteristic of *C. botulinum* type C or D or *C. novyi* type A.

Table 4.1: Isolates identified as Group III botulinum organisms: description of the animal and sample from which they were isolated.

Isolate ID	Source of isolate	Diagnosis	Sample type	BoNT/C directly present in sample	BoNT/C present after enrichment
Lch 1 **Lch 2** **Lch 11**	Horse (KW 1903)	AGS	Ileum contents	+	+
Lch 3 **Lch 4** **Lch 5**	Hare (KW 190)	Dysautonomia	S.I.[1] contents	+	-
Lch 6	Cat (LH10)	Dysautonomia	S.I. contents	+	+
Lch 7	Horse (LH18)	CGS	Faeces	-	-
Lch 8	Horse (LH14)	Recovered from CGS	Faeces	-	-
Lch 9	Horse (LH108)	Contact with EGS	Faeces	-	-
Lch 10	Horse (LH117)	Contact with EGS	Faeces	-	-
Lch 12	Horse (LH327A)	Recovered from CGS	Ileum contents	+	+
Lch 13	Horse (LH333A)	AGS	Ileum contents	-	+
Lch 14 **Lch 15**	Horse (LH 343A/B)	CGS	Ileum Faeces	NT[2] NT	NT NT
MPRL 3690	Horse	GS	Ileum	NT	NT

[1]S.I = small intestine
[2]NT= not tested

Table 4.2: GLC profiles of volatile fatty acids produce by the isolates. P= proprionic acid, B=butyric acid, A=acetic acid, V=valeric acid, L=lactic acid, S=succinic acid. Letters in upper case represent levels of fatty acids above 10 μmoles/ml, letters in lower case represent levels of fatty acids between 1-10 μmoles/ml, and letters in brackets represent levels between 0.2-1 μmoles/ml.

Isolate	Volatile fatty acids
Lch 1	pBv
Lch 2	pBv
Lch 11	pBv
Lch 3	pBv
Lch 4	pBv
Lch 5	pBv
Lch 6	pBv(l,s)
Lch 7	pBv
Lch 8	PB(v)
Lch 9	PBv(a)
Lch 10	Pba(v)
Lch 12	PBv(a)
Lch 13	PBv
Lch 14	PBv
Lch 15	PBv
MPRL 3690	PBv

4.1.2 Production of BoNT/C by isolates

The isolates were cultured anaerobically for five days at 30°C, and the culture supernatants were tested for the presence of BoNT/C by ELISA (as in Chapter 3). There was no detectable BoNT/C produced by any of the sixteen isolates. However, five of these organisms were isolated from samples in which BoNT/C had been detected either directly or after enrichment (Table 4.1).

4.1.3 Investigation of the surface antigens of Group III type strains and isolates

SDS-PAGE of EDTA-extracted surface antigens of isolates and Group III type strains

The surface antigens of the isolates were extracted with EDTA, separated by SDS-PAGE and stained with Coomassie blue to compare the surface protein profiles. The surface proteins of the isolates produced complex banding patterns on the gels (Fig. 4.1). However, there were similarities, observable by eye, between the isolates. The majority of isolates had three major bands – one observable near the top of the gel, one at 45-48kDa and one at 32-34kDa (indicated by arrows in Fig. 4.1) - and many minor bands. The second band was not as dominant in Ich 8 (lane 12) and the third band not as dominant in Ich 9, Ich 12 and MPRL 3690 (lanes 15-17) (Fig. 4.1). Isolates Ich 1, 3, 5, and 7 (lanes 2, 4, 6 and 7 respectively) had very similar banding patterns to each other, as did Ich 2 and 4 (lanes 3 and 5) (Fig. 4.1). Lch 11, 13 and 10 (lanes 8, 9 and 11) were also very similar; these isolates had a slightly higher molecular weight (MW) band at the top of the gel. Lch 14 and 15 (lanes 13 and 14) were almost identical and Ich 12 and MPRL 3690 (lanes 16 and 17) were very similar (Fig. 4.1). Lch 6 was run on a different gel (Fig. 4.2, lane 16) and has a different pattern of surface proteins from the other isolates.

The surface antigens of the Group III type strains were also extracted with EDTA and separated by SDS-PAGE. The Coomassie blue stained surface protein profiles of the type strains also produced complex banding patterns (Fig. 4.2); some similarities were observable. There are similarities in the surface protein banding patterns within *C. botulinum* type C type strains and *C. novyi* type A strains. *C. botulinum* type C strains MPRL 3922 and 2510 (lanes 2 and 3, Fig. 4.2) are very

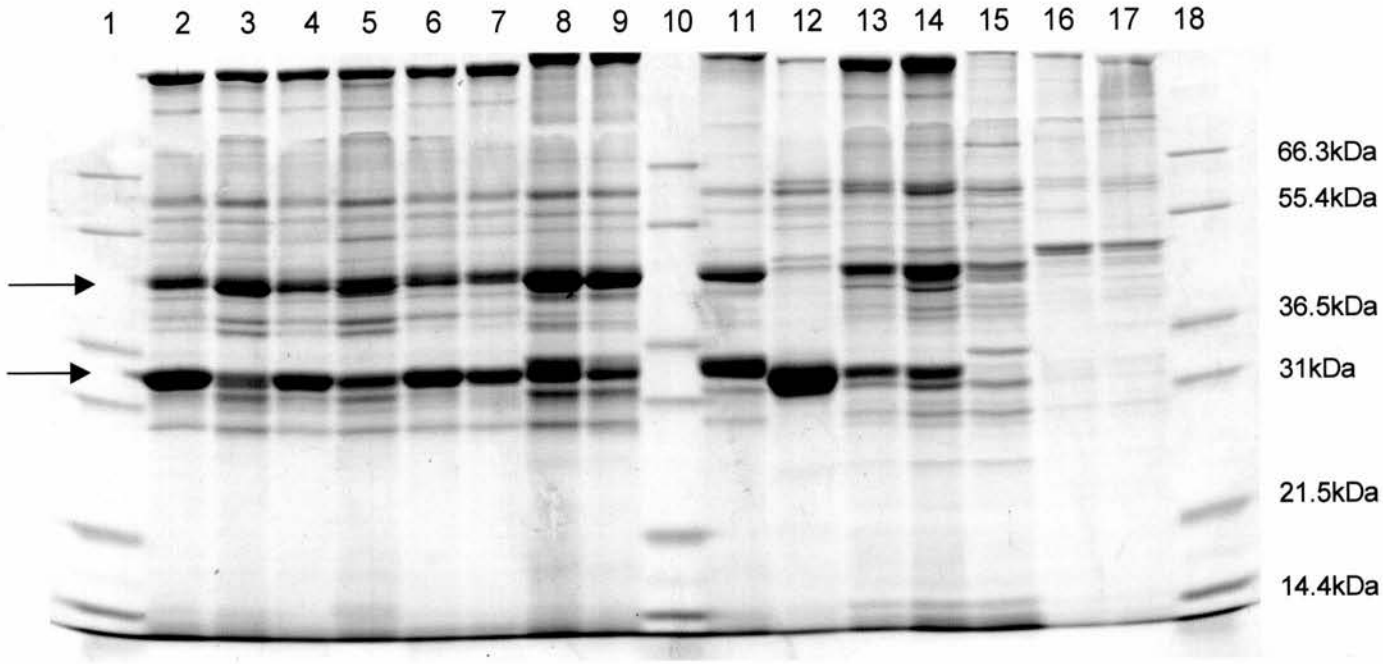


Figure 4.1: Coomassie stained SDS-PAGE gel of EDTA-extracted surface antigens. Lanes 1, 10 and 18, molecular weight markers; lane 2, Ich 1; lane 3, Ich 2; lane 4, Ich 3; lane 5, Ich 4; lane 6, Ich 5; lane 7, Ich 7; lane 8, Ich 11; lane 9, Ich 13; lane 11, Ich 10; lane 12, Ich 8, lane 13, Ich 15, lane 14, Ich 14; lane 15, Ich 9; lane 16, Ich 12; lane 17, MPRL 3690.
Upper arrow at ~45-48 kDa, lower arrow at ~32-34 kDa

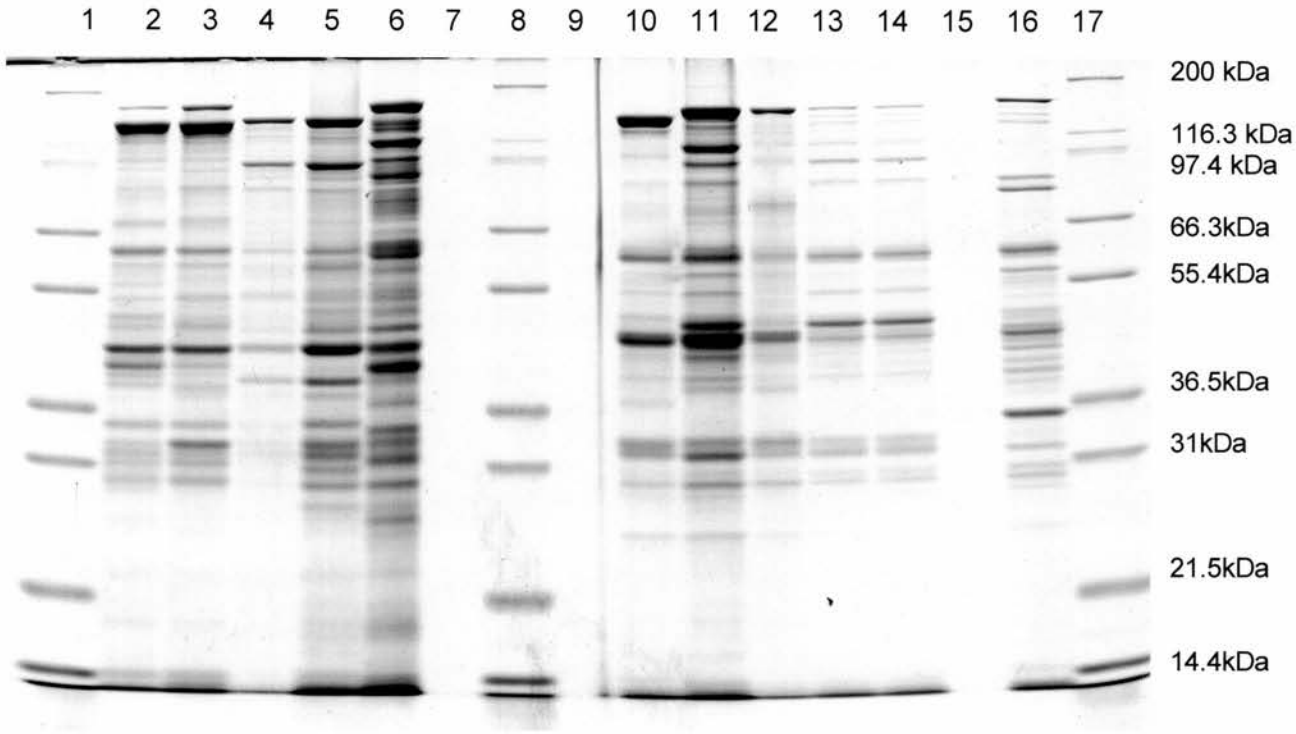


Figure 4.2: Coomassie stained SDS-PAGE gel of EDTA-extracted surface antigens. Lanes 1, 8 and 17, molecular weight markers; lanes 7, 9 and 15, single strength sample buffer; lane 2, MPRL 3922; lane 3, MPRL 2510; lane 4, MPRL 3493; lane 5, MPRL 4240; lane 6, MPRL 3923; lane 10, MPRL 2530; lane 11, MPRL 2531; lane 12, MPRL 2532; lane 13, MPRL 2533; lane 14, MPRL 2535; lane 16, Ich 6.

similar to each other, as are MPRL 3493 and MPRL 4240 (lanes 4 and 5, Fig. 4.2). With the *C. novyi* type A type strains, MPRL 2533 and MPRL 2535 (lanes 13 and 14, Fig.4.2) appear identical by surface antigen profile, and MPRL 2531 and MPRL 2532 (lanes 11 and 12) look very similar. However, The *C. novyi* type A type strain, MPRL 2534 (lane 6, Fig. 4.3), is not as similar to the other *C. novyi* type A strains. There are bands in common to the isolates and type strains, however the surface protein profiles of the isolates do not closely resemble those of the type strains. There are bands at 45-47kDa and 33-34kDa in all of the type strains. However, these do not appear as dominant as the bands in the isolates, particularly the band at 33-34kDa (Fig. 4.1 and 4.2). The surface protein profiles of the isolates appear to be more similar to each other than to the type strains.

SDS-PAGE of Group III surface antigens extracted with guanidine hydrochloride

Guanidine hydrochloride (GHCl) has been used to extract the S-layers of *Clostridium difficile* and other bacteria. The GHCl-extracted antigens of the Group III strains were separated by SDS-PAGE and stained with Coomassie blue. There were some minor differences in the Group III surface protein profiles when antigens were extracted with GHCl compared to EDTA (Fig. 4.3).

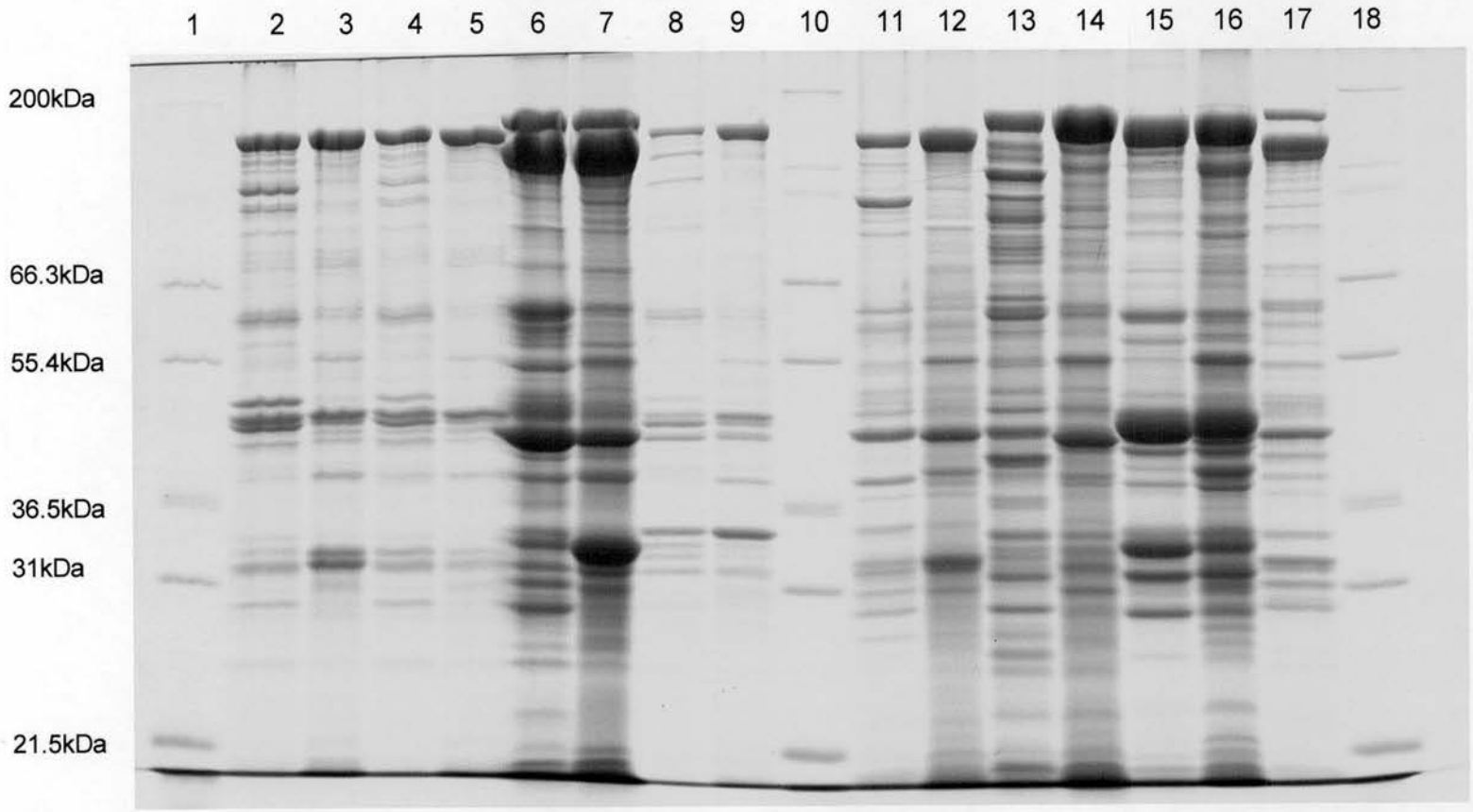


Figure 4.3: Coomassie stained SDS-PAGE gel of EDTA-extracted and GHCl-extracted surface antigens. Lanes 1, 10 and 18, molecular weight markers; lane 2, MPRL 2531 (EDTA); lane 3, MPRL 2531 (GHCl); lane 4, MPRL 2532 (EDTA); lane 5, MPRL 2532 (GHCl), lane 6, MPRL 2534 (EDTA); lane 7, MPRL 2534 (GHCl); lane 8, MPRL 2535 (EDTA); lane 9, MPRL 2535 (GHCl); lane 11, MPRL 4240 (EDTA); lane 12, MPRL 4240 (GHCl); lane 13, MPRL 3923 (EDTA); lane 14, MPRL 3923 (GHCl); lane 15, Ich 13 (EDTA); lane 16, Ich 13 (GHCl); lane 17, MPRL 2510 (EDTA).

Immunoblot of EDTA-extracted surface antigens with rabbit *C. novyi* antiserum

Group III type strains

The EDTA-extracted surface antigens of four type strains of *C. botulinum* type C (MPRL 2510, MPRL 3922, MPRL 4240, MPRL 3493), one type strain of *C. botulinum* type D (MPRL 3923) and five type strains of *C. novyi* type A (MPRL 2530, MPRL 2531, MPRL 2532, MPRL 2534, MPRL 2535) were separated by SDS-PAGE, transferred to nitrocellulose and reacted with antiserum raised against *C. novyi* type A (MPRL 2530). A major difference was observed between the *C. botulinum* type C/D and *C. novyi* type A strains. The antiserum recognised a major band at 46kDa in four of the five *C. novyi* type A type strains that was absent in the *C. botulinum* type C and D strains; a major band, at 43kDa, was recognised in the *C. botulinum* type C and D strains that was absent in four of the *C. novyi* type A strains (Fig. 4.4 and 4.5). One *C. novyi* type A type strain (MPRL 2534; lane 8, Fig. 4.4) had the 43kDa band and not the 46kDa band (the apparent weak band at 46kDa is due to spillage from lane 9). Other cross-reactions were observed with the antiserum binding to other surface antigens present in the Group III strains. However, the bands at 43kDa and 46kDa were the only consistent differences between the immunoblots of *C. novyi* type A and *C. botulinum* types C/D strains. These bands also reacted strongly with the antiserum, suggesting they were significant immunogenic antigens. This band difference was also observed when the surface antigens were extracted with guanidine hydrochloride (Fig. 4.5).

In the Coomassie stained gels, bands at both 46kDa and 43kDa were visible in MPRL 3922 and MPRL 2510 (*C. botulinum* type C strains), and MPRL 3923 (*C.*

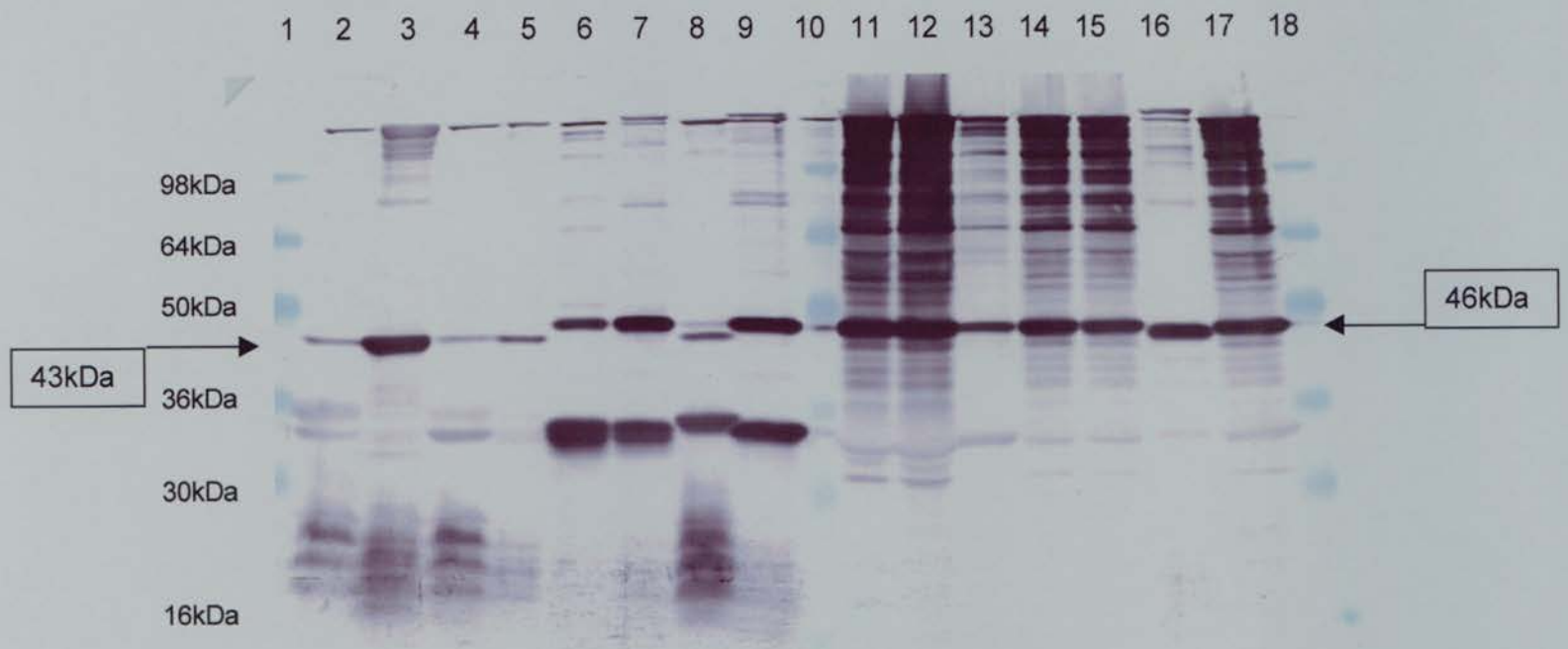


Figure 4.4: Immunoblot of EDTA-extracted surface antigens separated by SDS-PAGE and reacted with rabbit antiserum raised against *C. novyi* type A (MPRL 2530). Lanes 1, 10 and 18, molecular weight markers; lane 2, MPRL 3922; lane 3, MPRL 3493; lane 4, MPRL 2510; lane 5, MPRL 4240, lane 6, MPRL 2530; lane 7, MPRL 2531; lane 8, MPRL 2534; lane 9, MPRL 2532; lane 11, Ich 1; lane 12, Ich 2; lane 13, Ich 3; lane 14, Ich 4; lane 15, Ich 5; lane 16, Ich 6; lane 17, Ich 7.

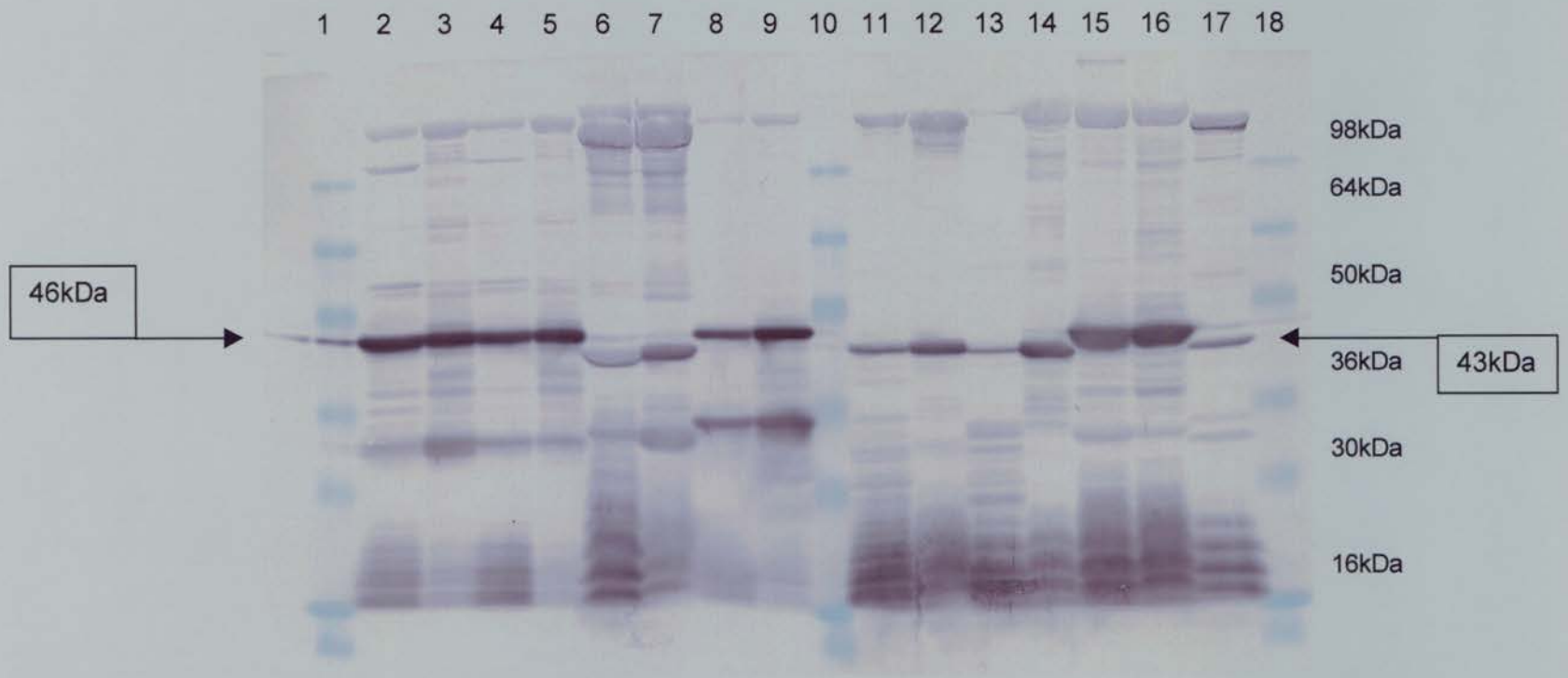


Figure 4.5: Immunoblot of EDTA-extracted and GHCl-extracted surface antigens separated by SDS-PAGE gel and blotted with rabbit antiserum raised against *C. novyi* type A (MPRL 2530). Lanes 1, 10 and 18, molecular weight markers; lane 2, MPRL 2531 (EDTA); lane 3, MPRL 2531 (GHCl); lane 4, MPRL 2532 (EDTA); lane 5, MPRL 2532 (GHCl), lane 6, MPRL 2534 (EDTA); lane 7, MPRL 2534 (GHCl); lane 8, MPRL 2535 (EDTA); lane 9, MPRL 2535 (GHCl); lane 11, MPRL 4240 (EDTA); lane 12, MPRL 4240 (GHCl); lane 13, MPRL 3923 (EDTA); lane 14, MPRL 3923 (GHCl); lane 15, Ich 13 (EDTA); lane 16, Ich 13 (GHCl); lane 17, MPRL 2510 (EDTA).

botulinum type D). Only the 43kDa band is recognised by the antiserum in the blot. However, MPRL 3493 and MPRL 4240 (*C. botulinum* type C strains) have a band at 46kDa but do not have a visible band at 43kDa in the Coomassie stained gel. However, these strains do have a band at 43kDa that is recognised in the immunoblot. The *C. novyi* type A strains have bands at 46-47kDa in the Coomassie stained gels and a number of fainter bands below this band, including bands at 43-44kDa. These bands are visible in the immunoblot, but the band at 46kDa is the dominant band recognised by the antiserum in these strains.

A ladder pattern is visible at the bottom of the immunoblot of the EDTA-extracted surface antigens of *C. botulinum* type C and D type strains and the *C. novyi* type A type strains (Fig. 4.4, lanes 2-9; Fig. 5, lanes 2, 4, 6, 8, 11, 13 and 17). This ladder pattern was also observable when the surface antigens were extracted with GHCl (Fig. 4.5, lanes 3, 5, 7, 9, 12 and 14). This ladder pattern is not observable in the Coomassie stained gels.

Group III animal isolates

The EDTA-extracted antigens of the 16 Group III isolates (from the horses, cat and hare) were also separated by SDS-PAGE and then transferred to nitrocellulose. The *C. novyi* type A antiserum recognised many surface antigens of these isolates and significantly bound to a major band at 46kDa, but not at 43kDa in all 16 isolates (Fig. 4.6). This suggested that they were more closely related to *C. novyi* type A strains than *C. botulinum* types C/D, based on the results of the immunoblots with the type strains.

There are major bands present on the Coomassie stained gels at around 46kDa in the majority of isolates; some isolates also have a band at around 44kDa, visible in

Figure 4.6: Immunoblot of EDTA-extracted surface antigens separated by SDS-PAGE and reacted with rabbit antiserum raised against *C. novyi* type A (MPRL 2530). Lanes 1, 10 and 18, molecular weight markers; lane 2, Ich 1; lane 3, Ich 2; lane 4, Ich 3; lane 5, Ich 4; lane 6, Ich 5; lane 7, Ich 7; lane 8, Ich 11; lane 9, Ich 13; lane 11, Ich 10; lane 12, Ich 8, lane 13, Ich 15, lane 14, Ich 14; lane 15, Ich 9; lane 16, Ich 12; lane 17, MPRL 3690.

the gel (Fig. 4.1), that is not as immunogenic as the band at 46kDa in the blot (Fig. 4.6). However, some isolates do not have a band at 46kDa – Ich 8, Ich 12 and MPRL 3690 – in the Coomassie stained gel (Fig. 4.1, lane 12, 16 an 17 respectively) and yet have a band at 46kDa in the immunoblot.

The ladder pattern observed at the bottom of the immunoblot in the type strains was also observed in some of the isolates. Lch 11, Ich 13, Ich 10, Ich 15, Ich 14, Ich 9 (Fig. 4.6, lanes 8, 9, 11, 13-15) all had ladder patterns that reacted strongly with the anti-novyi serum; weakly reacting ladder patterns may be present in Ich 8 (lane 12), Ich 12 (lane 16) and MPRL 3690 (lane 17). However, Ich 1-7 (Fig. 4.6, lanes 2-7 and Fig. 4.4, lane 16) did not have a visible ladder pattern in the immunoblot. The isolates could therefore be grouped into two groups on the basis of the presence of this ladder pattern.

Immunoblot of EDTA-extracted surface antigens with equine serum

The EDTA-extracted surface antigens of Group III type strains and isolates were blotted with several different equine serum samples. The distinctive band difference between *C. botulinum* type C/D and *C. novyi* type A strains that was observed with the rabbit antiserum raised against *C. novyi* type A was not apparent when equine serum was used. When equine serum sample LH341c (from a horse with AGS) was used, it bound to bands at approximately 46kDa and 43kDa in both *C. botulinum* type C/D type stains and *C. novyi* type A type stains (Fig. 4.7a and b). However, the profile of bands reacting with equine serum varied between serum samples. When serum sample LH 214c (from a horse with AGS) was used, it bound to a 48kDa and 44kDa band present in the *C. novyi* type A surface antigens (MPRL 2532), but not at 46kDa (Fig. 4.8a); this serum sample recognised both a 46kDa and 43kDa band in a *C. botulinum* type C type strain (MPRL 2510) (Fig. 4.8a). Serum sample S48,

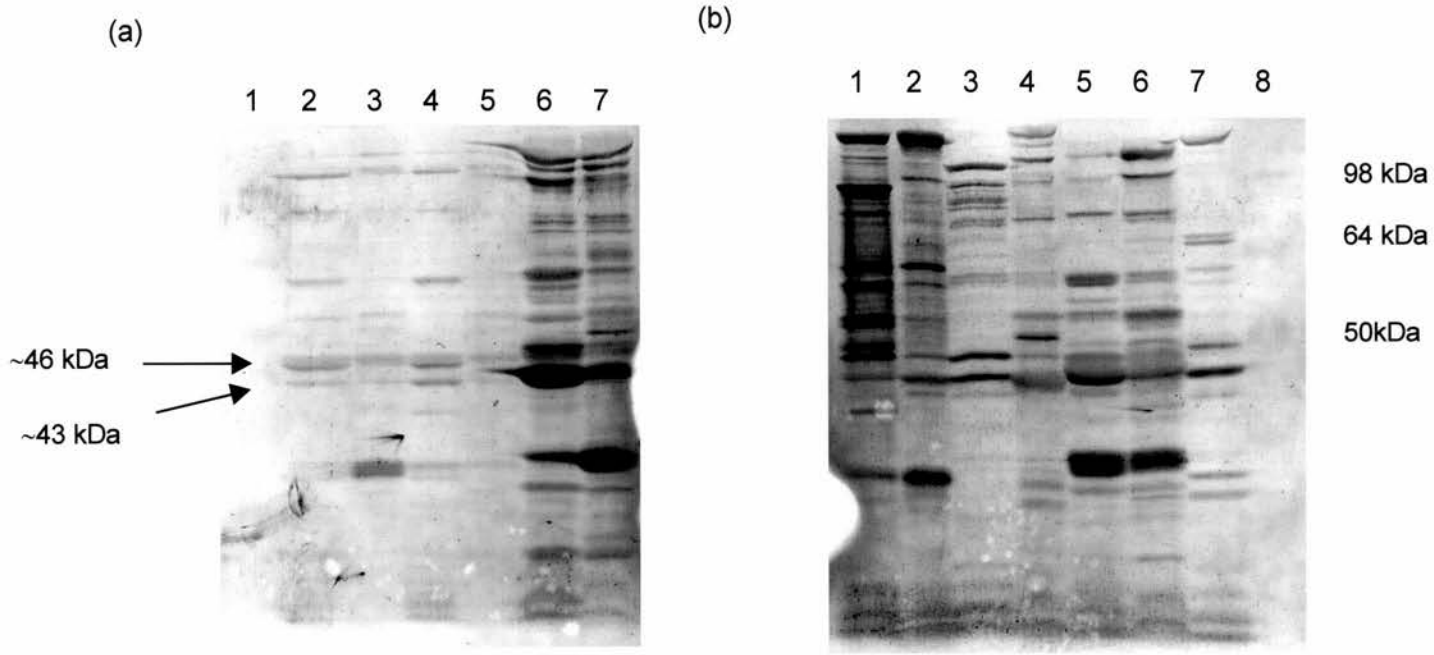


Figure 4.7: Immunoblot of EDTA-extracted and GHCl-extracted surface antigens separated by SDS-PAGE and blotted with equine serum, LH 341c.

(a) Lane 1, molecular weight markers; lane 2, MPRL 2531 (EDTA); lane 3, MPRL 2531 (GHCl); lane 4, MPRL 2532 (EDTA); lane 5, MPRL 2532 (GHCl); lane 6, MPRL 2534 (EDTA); lane 7, MPRL 2534 (GHCl).

(b) Lane 1, MPRL 4240 (EDTA); lane 2, MPRL 4240 (GHCl); lane 3, MPRL 3923 (EDTA); lane 4, MPRL 3923 (GHCl); lane 5, Ich 13 (EDTA); lane 6, Ich 13 (GHCl); lane 7, MPRL 2510 (EDTA); lane 8, molecular weight markers

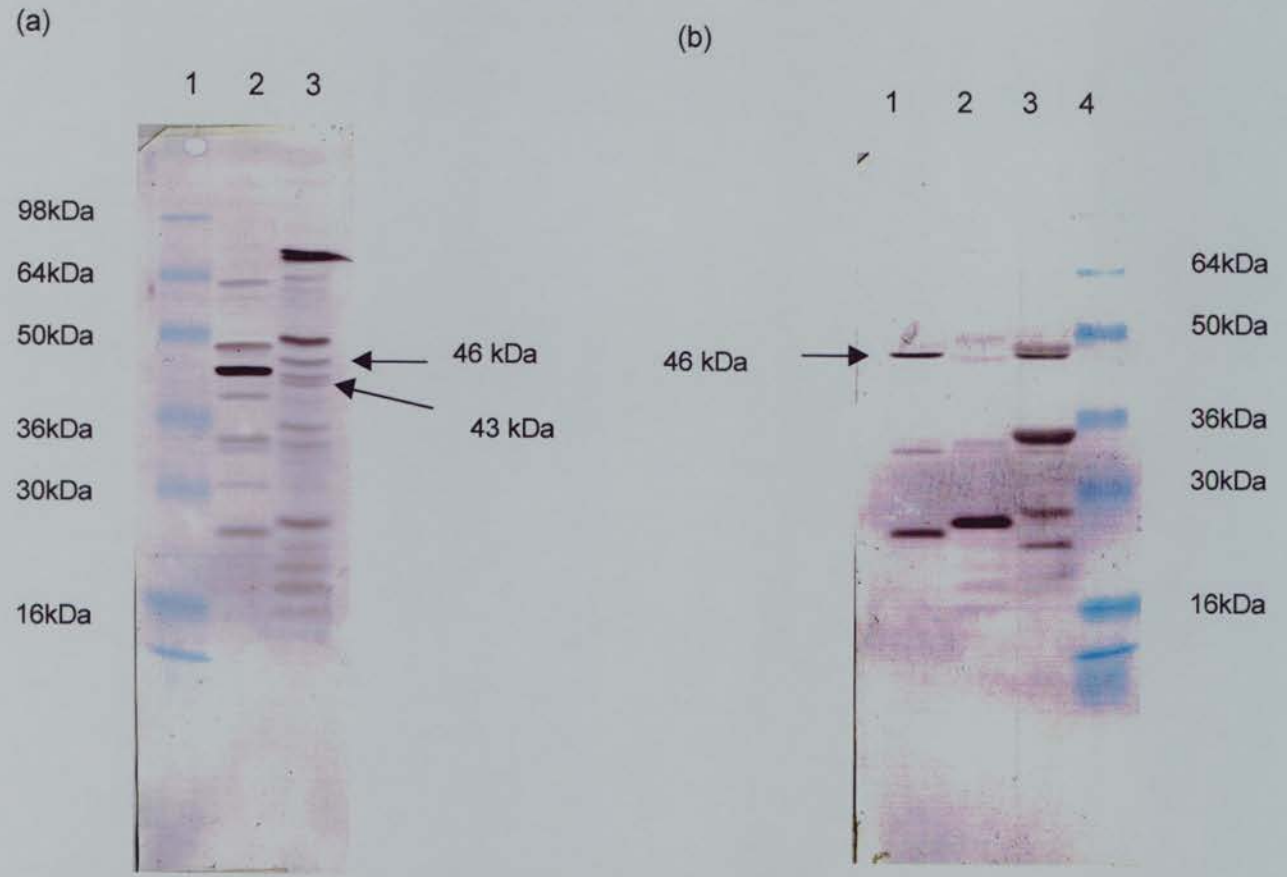


Figure 4.8: Immunoblot of EDTA-extracted surface antigens separated by SDS-PAGE and blotted with two different equine serum samples.

(a) Blotted with equine serum sample LH 214c: lane 1, molecular weight markers; lane 2, MPRL 2532; lane 3, MPRL 2510.

(b) Blotted with equine serum sample S48: lane 1, MPRL 2532; lane 2, MPRL 2510; lane 3, Ich 7; lane 4, molecular weight markers.

collected from the horse with CGS from which isolate Ich 7 had been isolated, recognised a band at 46kDa in the surface antigens of both the *C. novyi* type A and Ich 7, and a band at 48kDa the *C. botulinum* type C (Fig. 4.8b). In addition this serum sample bound to surface antigens at 34, 28, and 24kDa in the isolate; these three bands were at different molecular weights to those recognised in either the *C. botulinum* type C or *C. novyi* type A.

4.1.2 Investigation of Group III toxin genes by PCR

PCR detection of the novyi alpha toxin gene

Three out of five *C. novyi* type A type strains carried the alpha toxin gene (Table 4.3), as demonstrated by the production of a 260bp PCR product, using the NOVA primers (Fig. 4.9). The lack of PCR product in the other two type strains suggested that the phage carrying the alpha toxin gene had been lost. PCR of DNA from the animal isolates, with the NOVA primers, produced a 260bp product in three out of the 16 isolates, Ich 14, Ich 15, and MPRL 3690 (Table 4.4).

PCR detection of the BoNT/C and BoNT/D genes

PCR with the CS primers did not produce a product with any of the type strains or isolates, including the positive control, a *C. botulinum* type C_{α} type strain (MPRL 3922). However, subsequent analysis of the culture supernatants of the type strains for the presence of BoNT/C by ELISA confirmed that none of the type strains were producing BoNT/C. The isolates also did not produce BoNT/C when tested by ELISA. Although the results of the PCR could not be confirmed due to the lack of a positive control, the ELISA results would suggest that none of the type strains or isolates were carrying the BoNT/C gene. These results suggest that the *C. botulinum* type C_{α} strain had lost the phage carrying the BoNT/C gene.

PCR with the DS primers produced a 497bp product (Fig. 4.10) from DNA of *C. botulinum* type D type strain (NCTC 3923: lane 7) demonstrating the presence of the BoNT/D gene. None of the other Group III type strains or isolates were carrying the BoNT/D gene (Tables 4.3 and 4.4).

Table 4.3: Toxin genes detected in type strains by PCR and their identity by immunoblot. CS= primers for BoNT/C, DS= primers for BoNT/D, C2CI = primers for component I of the C2 toxin, C2CII = primers for component II of the C2 toxin, NOVA = primers for *C. novyi* alpha toxin, BAC = primers for bacterial 16S rRNA.

Lab ID	Organism	Positive PCR products amplified by primer pairs						Identity by blot
		CS	DS	C2CI	C2CII	NOVA	BAC	
MPRL 3922	***C. botulinum* type C_α**	-	-	+	+	-	+	*C. botulinum* type C/D
MPRL 2510	***C. botulinum* type C_β**	-	-	+	+	-	+	*C. botulinum* type C/D
MPRL 3493	***C. botulinum* type C**	-	-	-	-	-	+	*C. botulinum* type C/D
MPRL 4240	***C. botulinum* type C**	-	-	-	+	-	+	*C. botulinum* type C/D
MPRL 3923	***C. botulinum* type D**	-	+	+	-	-	+	*C. botulinum* type C/D
MPRL 2530	***C. novyi* type A**	-	-	-	-	+	+	*C. novyi* type A
MPRL 2531	***C. novyi* type A**	-	-	-	-	+	NT	*C. novyi* type A
MPRL 2532	***C. novyi* type A**	-	-	+	-	+	NT	*C. novyi* type A
MPRL 2533	***C. novyi* type A**	-	-	-	-	-	+	*C. novyi* type A
MPRL 2534	***C. novyi* type A**	-	-	+	-	-	NT	*C. botulinum* type C/D
MPRL 2535	***C. novyi* type A**	NT[1]	NT	NT	NT	NT	NT	*C. novyi* type A

[1] NT- not tested

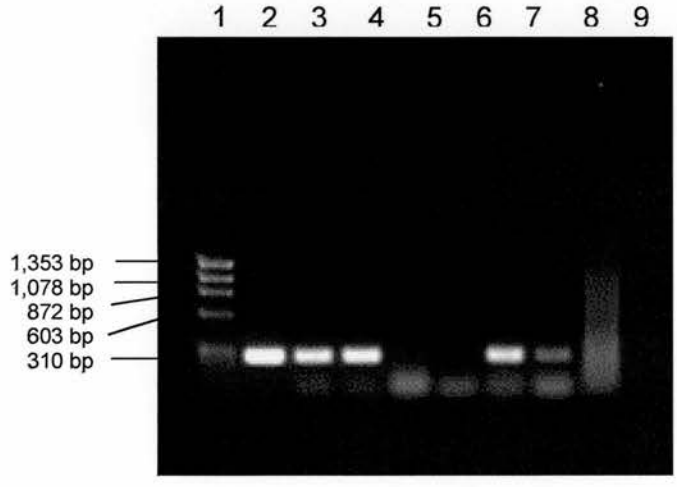


Figure 4.9: Detection of a 260bp PCR product demonstrating the presence of the *C. novyi* type A alpha toxin gene in MPRL 2530, MPRL 2531 and Ich 14 and 15. Lane 1, ϕX174 Hae III digest; lane 2, MPRL 2530 (standard); lane 3, MPRL 2530 (miniprep), lane 4, MPRL 2531; lane 5, MPRL 2533; lane 6, Ich 9; lane 7, Ich 14; lane 8, Ich 15; lane 9, no template DNA.

Table 4.4: Toxin genes detected by PCR in animal isolates and the identity of the isolates by immunoblot. Abbreviations as for Table 4.3.

Lab ID	Positive PCR products amplified by primer pairs						Identity by blot
	CS	DS	C2CI	C2CII	NOVA	BAC	
Lch 1	-	-	+	-	-	+	*C. novyi* type A
Lch 2	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 11	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 3	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 4	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 5	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 6	-	-	+	-	-	NT[2]	*C. novyi* type A
Lch 7	-	-	+	-	-	NT	*C. novyi* type A
Lch 8	-	-	+[1]	-	-	NT	*C. novyi* type A
Lch 9	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 10	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 12	-	-	+[1]	-	-	+	*C. novyi* type A
Lch 13	-	-	+	-	-	NT	*C. novyi* type A
Lch 14	-	-	+[1]	-	+	+	*C. novyi* type A
Lch 15	-	-	+[1]		+	+	*C. novyi* type A
MPRL 3690	-	-	+[1]	-	+	+	*C. novyi* type A

[1] PCR products were amplified again to get a positive PCR result [2] NT = not tested

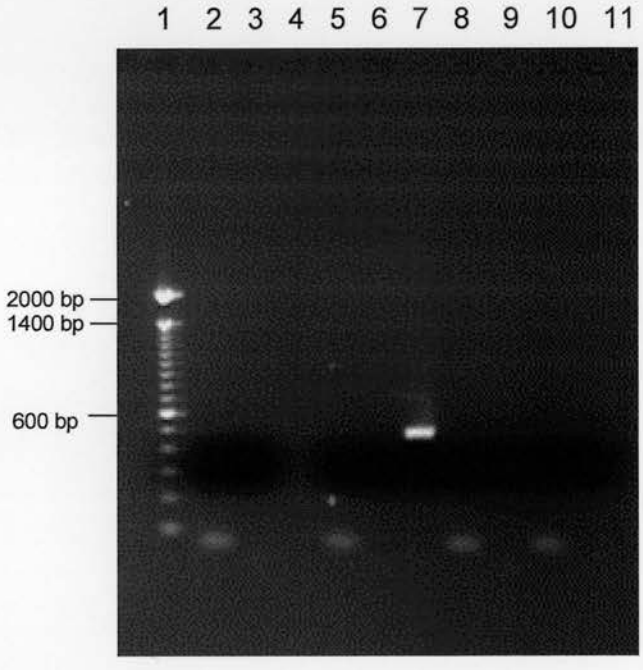


Figure 4.10: Detection of a 497 bp PCR product demonstrating the presence of the *C. botulinum* type D neurotoxin gene in MPRL 3923. Lane 1, 100bp DNA ladder; lane 2, MPRL 3922 with CS11/CS22; lane 3, MPRL 3922 with DS11/DS22; lane 4, no template DNA and no primer; lane 5, MPRL 2510 with CS 11/CS22; lane 6, MPRL 2510 with DS11/DS22; lane 7, MPRL 3923 with DS11/DS22; lane 8, no template DNA with CS11/CS22; lane 9, no template DNA with DS11/DS22, lane 10, MPRL 3922 with no primers; lane 11, MPRL 2510 with no primers.

PCR detection of C2 toxin gene

PCR with the C2CI primers amplified a 310bp (Fig. 4.11) product in two of the four *C. botulinum* type C type strains (MPRL 3922 and MPRL 2510), in the *C. botulinum* type D type strain (MPRL 3923) and in two of the five *C. novyi* type A type strains (MPRL 2532 and MPRL 2534) demonstrating the presence of the component I gene of the C2 toxin in these organisms (Table 4.3). The component I gene of the C2 toxin was also detected in all sixteen of the animal isolates (Table 4.4). Two rounds of amplification were required to detect the 310bp product in 12 of these isolates (Table 4.4).

PCR with the C2CII primers produced a 1093bp product (Fig. 4.12) with DNA from three *C. botulinum* type C type strains (MPRL 3922, MPRL 2510, and MPRL 4240), demonstrating the presence of the component II gene of the C2 toxin in these organisms (Table 4.3). Two of these type strains also carried the component I gene for the C2 toxin (MPRL 3922 and MPRL 2510). The *C. novyi* type A strains or the isolates that had been shown to have the component I gene of the C2 toxin did not produce a product when amplified with the primers for the component II gene (Tables 4.3 and 4.4).

PCR for bacterial 16S rRNA

The BAC primers detected universal bacterial DNA by amplifying a 763bp product from a conserved region of the gene encoding 16s rRNA (Fig. 4.13). MPRL 2533 and MPRL 3493 did not produce PCR products when amplified with any of the primers for toxin genes, but did produce a 763bp product when amplified with the BAC primers (Table 4.3). Therefore, the BAC primers were used to confirm that the negative results obtained for these type strains, with the primers for the toxin genes, were not due to the presence of inhibitors. In addition, the BAC primers amplified a

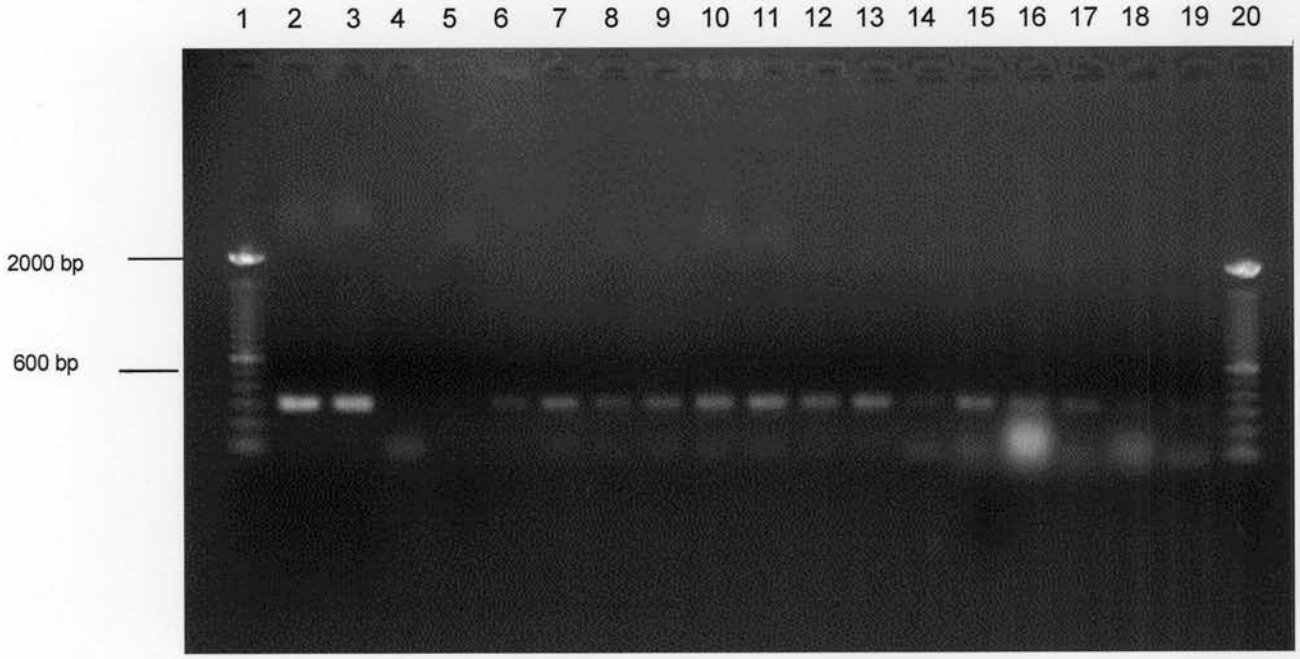


Figure 4.11: Detection of 310 bp PCR product demonstrating the presence of the component I gene of the C2 toxin in MPRL 3922, MPRL 2510, MPRL 3923, Ich 9, Ich 10, Ich 11, Ich 12, Ich 13, Ich 14, Ich 15, MPRL 3690, Ich 2, Ich 3, and Ich 4. Lanes 1 and 20, 100 bp DNA ladder; lane 2, MPRL 3922; lane 3, MPRL 2510; lane 4, MPRL 3493; lane 5, MPRL 4240; lane 6, MPRL 3923; lane 7, Ich 9; lane 8, Ich 10; lane 9, Ich 11; lane 10, Ich 12; lane 11, Ich 13; lane 12, Ich 14; lane 13, Ich 15; lane 14, MPRL 3690; lane 15, Ich 2; lane 16, Ich 3; lane 17, Ich 4; lane 18, Ich 6; lane 19, no template DNA.

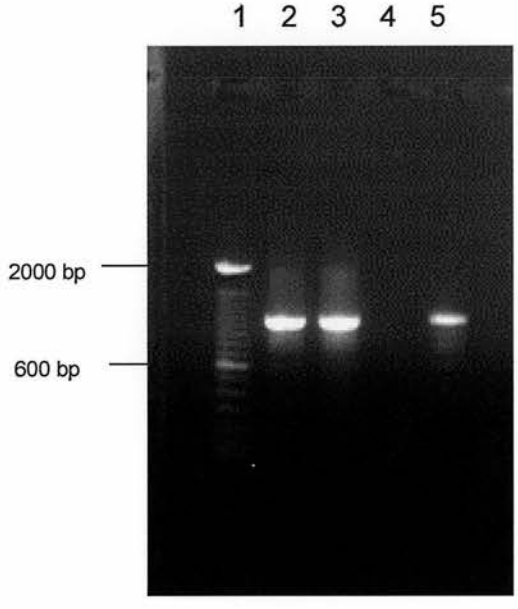


Figure 4.12: Detection of 1093 bp PCR product demonstrating the presence of the component II gene of the C2 toxin in MPRL 3922, MPRL 2510, and MPRL 4240. Lane 1, 100bp DNA ladder; lane 2, MPRL 3922; lane 3, MPRL 2510; lane 4, MPRL 3493; lane 5, MPRL 4240.

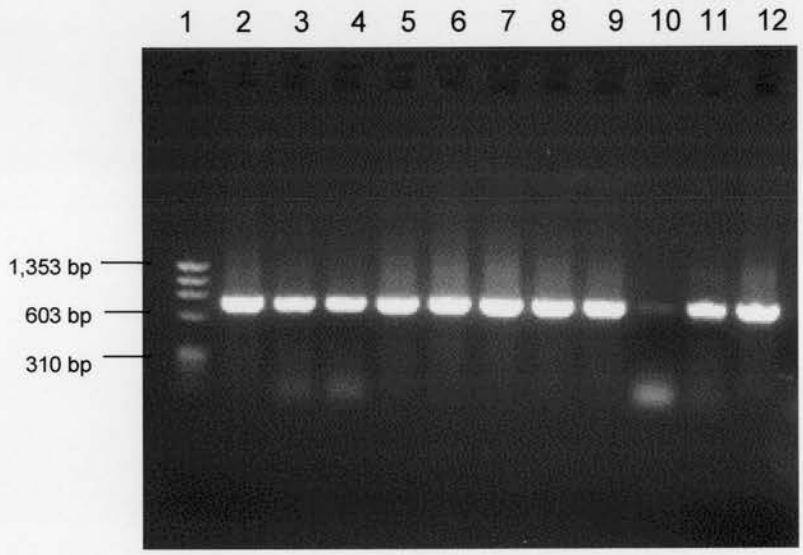


Figure 4.13: Detection of a 763 bp PCR product demonstrating the presence of the gene for bacterial 16sRNA. Lane 1, ϕX174 Hae III digest; lane 2, MPRL 3923; lane 3, MPRL 4240; lane 4, MPRL 2533; lane 5, Ich 11; lane 6, Ich 2; lane 7, Ich 3; lane 8, Ich 4; lane 9, Ich 5; lane 10, Ich 9; lane 11, Ich 10; lane 12, Ich 12.

763bp product from type strains MPRL 3922, MPRL 2510, MPRL 4240, MPRL 3923, and MPRL 2530 (Table 4.3) and isolates Ich 1-5, 9-12, 14-15 and MPRL 3690 (Table 4.4).

Negative controls

When no template DNA was added to the reaction mixture, no PCR products were obtained. The negative controls also remained negative when there was more than one round of amplification i.e. when products were re-amplified.

4.2 Discussion

Sixteen isolates identified as botulinum Group III organisms were isolated from equine, leporine and feline gastrointestinal samples after enrichment. The standard methods used in the identification of anaerobic bacteria such as morphology, biochemical profiles and GLC cannot distinguish between *C. botulinum* types C and D or *C. novyi* type A (Group III organisms). Identification of these organisms to the species level requires demonstration of their ability to produce the type specific toxin – BoNT/C for *C. botulinum* type C, BoNT/D for *C. botulinum* type D and the *C. novyi* alpha toxin for *C. novyi* type A – each of which is encoded by a separate bacteriophage. None of the isolates produced BoNT/C in vitro, despite five of the organisms being isolated from samples in which BoNT/C had been detected either directly or after enrichment. The bacteriophages encoding the toxin genes are pseudolysogenic, consequently they are unstable and are easily lost from the host bacterial cell. Sporulation, subculturing in the laboratory and growth of bacteria at

temperatures of 37°C can all predispose to loss of the phage (Eklund et al, 1987). Repeated subculturing was required to isolate these organisms from the intestinal samples. There is no selective media for the Group III organisms and they are very fastidious, making isolation from GI samples very difficult, as they are usually overgrown by other bacteria present in the sample. In this study, many of the samples after plating out were incubated at 37°C in the anaerobic cabinet due to difficulties in handling large numbers of plates in gas jars. It is therefore possible that some of these isolates were producing BoNT/C in vivo but had lost the phage carrying the BoNT/C gene by the time they were isolated in pure culture.

No PCR products were obtained when DNA from the isolates were amplified with primers for the BoNT/C and BoNT/D genes, thus demonstrating that the isolates were not carrying either the bacteriophage encoding the BoNT/C gene or the BoNT/D gene. However, the positive control did not give a PCR product with primers for the BoNT/C gene so there is a possibility that this PCR reaction did not work; it was thought that the *C. botulinum* type Cα type strain used as the positive control had lost the bacteriophage. This was subsequently confirmed by a negative toxin result by ELISA. PCR demonstrated the presence of the *C. novyi* alpha toxin gene in three of the isolates (Ich 14, 15 and MPRL 3690). The presence of the gene for the alpha toxin, in conjunction with the GLC profile, microbiological and biochemical properties of this organism, enabled the identification of these three organisms as *C. novyi* type A. Based on the standard identification methods, the other 13 isolates are not identifiable any further than Group III organisms as they are no longer carrying the major species-specific toxin genes.

Immunoblotting of the surface antigens of Group III organisms, with antiserum raised against *C. novyi* type A, presented a novel method for distinguishing between *C. botulinum* type C/D and *C. novyi* type A. The antiserum bound to a major band at 46kDa in *C. novyi* type A type strains and to a major band at 43kDa in the *C. botulinum* type C and D strains; the 46kDa band was not recognised in the *C. botulinum* type C/D strains and the major band at 43kDa band was not recognised in the *C. novyi* type A strains. Application of this immunoblot method to the surface antigens of the isolates demonstrated the presence of the 46kDa band in all 16 isolates, suggesting that they were *C. novyi* type A strains as opposed to *C. botulinum* type C or D. This enabled the identification of the remaining 13 isolates as *C. novyi* type A; three had already been identified as *C. novyi* type A through possession of the alpha toxin gene.

The ladder pattern, observed at the bottom of the immunoblots of the *C. botulinum* type C/D and *C. novyi* type A type strains, is similar in appearance to the ladder pattern produced by lipopolysaccharide from Gram-negative organisms. The ladder pattern in these blots, not observable in the Coomassie stained gels, is probably produced by a lipocarbohydrate from the cytoplasmic membrane (Poxton, 1984); EDTA treatment has been shown to solubilise the membrane carbohydrates from *Clostridium difficile* and *Clostridium sordelli* (Poxton and Cartmill, 1982). Eight of the animal isolates did not have a visible ladder pattern in the blot. The membrane-bound lipocarbohydrate is either absent in these isolates, or more likely it is not cross-reactive with the anti-novyi serum that was used in this immunoblot, suggesting at least two "serotypes" of this molecule.

It is difficult to compare directly bands present on the immunoblot with those visible on the Coomassie stained SDS-PAGE gels, due to the complexity of the surface

protein profiles and possible differences between the molecular weight standards used for calibration of the bands in the gel and in the blot. There are many bands visible on the Coomassie stained gel in the region of interest i.e. approximately between 41 and 49kDa, and some of these bands differ in size by only 1kDa. Therefore it is difficult to be certain that the band at 46kDa in the immunoblot is the same antigen as the band calculated as having a molecular weight of 46kDa in the gel. Despite these uncertainties, several important observations can be made. *C. botulinum* type D and two of the type C strains had bands present at both 46kDa and 43kDa in the gel but only the band at 43kDa was recognised by the rabbit antiserum in the blot. These results suggest that the band at 46kDa in the *C. botulinum* type C and D type strains is not immunologically cross-reactive with any antigen present in the *C. novyi* type A type strain MPRL 2530, that was used to prepare the antiserum used in this blot. However, the band at 43kDa in the botulinum strains is immunologically cross-reactive with an antigen present in the *C. novyi* type A strain. Conversely the other two *C. botulinum* type C type strains did not have a band visible at 43kDa in the gel but did have a band at this molecular weight in the immunoblot. It is possible that this band was only a minor surface protein in these strains but strongly cross-reactive; the Coomassie blue may not have been sufficiently sensitive to stain the band in these strains.

The rabbit antiserum reacted strongly with both the band at 46kDa and the band at 43kDa in the immunoblot, suggesting that these bands were strongly immunogenic. It could be speculated that the immunogenic bands at 43kDa and 46kDa in the different species represent a similar antigen but with a slight difference in molecular weight.

When equine serum was used in blots against the surface antigens, all three of the serum samples used bound to surface antigens present in both *C. botulinum* type C and *C. novyi* type A, suggesting natural previous exposure to these organisms. However, the consistent difference in molecular weight of the major immunogenic band between *C. botulinum* type C/D and *C. novyi* type A was not observed when equine serum was used. In fact, two of the equine serum samples bound to antigens at both 43 and 46kDa in *C. botulinum* type C strain; one of these serum samples also bound to antigens at both these molecular weights in *C. novyi* type A. These results may reflect that the horses have been previously exposed to both *C. botulinum* type C and *C. novyi* type A, thereby making immune responses to antigens which may not necessarily be cross-reactive between these species. One of the serum samples did not bind to a band at 46kDa in the *C. novyi* type A but bound to bands at 43kDa and 46kDa in the *C. botulinum* type C: it could be speculated that this horse may have only been exposed to *C. botulinum* type C previously. Another consideration may be that the horses have been previously exposed to other cross-reactive antigens that have enabled recognition of these surface antigens; horses may also respond to different antigens than the rabbit that was used to prepare the antiserum. Clearly there were differences in the particular surface antigens that were recognised by the antibodies in the different equine serum samples, indicating differences in exposure to these strains, or crossreactive antigens and possibly also differences between individual horses to respond to particular antigens. All three equine serum samples investigated bound to a 46kDa and/or a 43kDa surface antigen in *C. botulinum* type C and/or *C. novyi* type A, indicating that these antigens may be significant immunogens.

The Group III organisms do not appear to have regular crystalline surface proteins (S-layers) that have been described in many other bacterial species, including

Clostridium difficile (Kawata et al, 1984). S-layers are the outermost component of the cell envelope and are generally composed of one or two protein or glycoprotein molecules that can assemble into a 2D lattice structure. High concentrations of guanidine hydrochloride are used to extract the protomeric subunits of the S-layers from the cell wall, which should appear as a single band when run on a gel. However, when the group III strains were extracted with GHCl the surface protein profiles were both complex and very similar to the protein profiles obtained when these cells were extracted with EDTA. Periodic structures, thought to be S-layers have been described in *C. botulinum* (Takagi et al, 1965, cited in Sleytr and Messner, 1983) and *C. novyi* (Schallen and Wacke, 1974, cited in Sleytr and Messner, 1983) but the lattice has not been further characterised. S-layers on pathogenic bacteria are of interest as potential virulence factors and have been implicated in a wide range of functions. S-layers can act as a physical barrier to invasion; some can protect the cell from muramidases, proteases, the host immune response or from bacteriophages, conversely, others can act as specific receptors for bacteriophages; some have been involved in adhesion to epidermal surfaces (Sleytr and Messner, 1983). Different S-layers have been shown to have different functions: the S-layers of *C. botulinum* and *C. novyi* are thought to prevent the release of macromolecules from the cells, resulting in intracellular accumulation of toxins (Sleytr and Messner, 1983). However, these S-layers could not be characterised by the GHCl extraction method used in this study.

PCR was used to determine how many of the Group III type strains carried the C2 toxin gene and whether it could be found on the *C. novyi* type A strains. The C2 toxin is produced by the majority of *C. botulinum* type C strains and some *C. botulinum* type D strains (Jansen, 1971), but there are no reports of the C2 toxin being produced by *C. novyi* type A. It was thought that the detection of the C2 toxin

gene could provide a useful alternative method for differentiating between *C. botulinum* type C and *C. novyi* type A. However, the identification of the Group III organisms was further complicated by the detection of the C2 toxin component I gene in a *C. novyi* type A type strain (MPRL 2532) that also carried the *C. novyi* alpha toxin gene and in all 16 of the isolates identified as *C. novyi* type A by the immunoblot method. The presence of the C2 gene on *C. novyi* type A strains may be evidence of genetic transfer from *C. botulinum* type C or D strains in vivo; the C2 gene may be on a mobile genetic element. The presence of the C2 gene in a *C. novyi* type A type strain that is also carrying the alpha toxin gene further demonstrates the close relationships between the Group III organisms. However, the C2 toxin is a binary toxin, requiring both components I and II to function; the gene for component II was not identified in the *C. novyi* type strains or in the isolates. ADP-ribosyltransferase activity is associated with component I, and binding to the cell surface receptor is associated with component II. It is not known whether the strains and isolates in which only the component I gene could be detected, are capable of producing active C2 toxin.

The component I gene was detected in only two of the *C. botulinum* type C type strains and in the *C. botulinum* type D strain. The component II gene was also detected in these two type C strains and in another type C strain that did not have the component I gene. These results suggest that only two of the four type C type strains (MPRL 3922 and MPRL 2510) can produce the C2 toxin. However, the other two type C type strains (MPRL 3493 and MPRL 4240) had been previously classified as C2 toxin producers – MPRL 3493 is a NCTC type Cβ strain and MPRL 4240 has been used to produce C2 toxin by other workers (Ohishi et al, 1980; Ohishi, 1983). It is possible that there are variations in the CI and CII gene

sequences between organisms preventing amplification by the PCR primers. However, the primers for the component I gene had previously been shown to amplify a 310bp product from MPRL 4240 (Fujii et al, 1996). Heterogeneity in the structure of component II has been reported (Ohishi and Okada, 1986; Ohishi and Hama, 1992), as has genetic heterogeneity in the component II gene (Kimura et al, 1998). Strains have been divided into three groups based on differences in molecular weight and biological activity of the C2 toxin; these differences are thought to be due to differences in component II (Ohishi and Okada, 1986). However, the primer sets chosen to amplify the component II gene in this study had been shown to amplify DNA from all three of these groups (Kimura et al, 1998). Another possibility is that the component I and II genes are on mobile elements and have been lost from these type C strains. It is known that these genes are not on bacteriophages but it is theoretically possible that they are on a transposable element that has been inserted into the chromosome. Loss of C2 toxin production by a strain during laboratory culture led Eklund et al (1987) to hypothesise that the C2 toxin may be encoded on a plasmid.

The investigation of C2 toxin production, in type strains with either one of the two component genes or neither of the component genes detected by PCR, is required to establish whether these genes are no longer present or whether the PCR is not optimised for the detection of the genes in all the type strains. Ideally, the isolates also need to be tested for their ability to produce active C2 toxin; the reagents to do this are not available at present.

One *C. novyi* type A type strain (MPRL 2534) did not fit with the immunoblot method of identification: the rabbit antiserum reacted with a band at 43kDa and not 46kDa. On this basis the organism should be identified as *C. botulinum* type C/D.

Furthermore, PCR did not detect the *C. novyi* alpha toxin gene but did detect the C2 toxin component I gene. It is possible that this isolate was originally incorrectly identified as a non-toxigenic *C. novyi* type A, when actually it was a non-neurotoxigenic *C. botulinum* type C/D. On the basis of the toxin producing results, the organism can only be identified as a Group III organism; the immunoblot method identifies it as *C. botulinum* type C or D. This organism is not a NCTC strain, but an isolate from the laboratory culture collection, and its source is unknown (it was isolated in 1972).

There is some question as to whether the Group III organisms should be classified as separate species. Previously they have only been differentiated from each other on the basis of the production of a major toxin that is encoded by a bacteriophage. Not only are these bacteriophages pseudolysogenic, but they can also potentially infect all three separate species, causing the organisms to produce different toxins and effectively changing the identity of that organism. Previously, minor toxins produced by these organisms could be used as an extra tool to differentiate between them, such as the gamma toxin produced by *C. novyi* type A and the C2 toxin produced by the majority of *C. botulinum* type C and D. However, this study has demonstrated the presence of the C2 toxin gene in a *C. novyi* type A type strain that is also carrying the alpha toxin gene, highlighting even further how closely related these organisms are. However, this study has also demonstrated that there are antigenic differences in the surface antigens of *C. botulinum* type C/D and *C. novyi* type A.

Only 16 Group III organisms were isolated from samples of small intestinal contents from 77 animals (48 with dysautonomia and 29 without) and from the faeces of 117 animals (45 with dysautonomia and 72 without). The identification of all these

isolates as *C. novyi* type A does not preclude them from being the organisms that were producing BoNT/C in vivo. It has been shown that Group III organisms can be infected simultaneously with the BoNT/C phage and alpha toxin phage resulting in co-expression of BoNT/C and *C. novyi* alpha toxins. It is thought that this also happens in vivo but is not identified due to loss of one of the phages during laboratory isolation; the carriage of two of these phages is an even more unstable state. An organism producing BoNT/C was isolated from a broiler chicken with toxicoinfectious botulism and originally identified as *C. botulinum* type C; subsequent detection of small quantities of the *C. novyi* γ toxin suggested that the organism was actually a *C. novyi* type A infected with the BoNT/C phage (Eklund et al, 1987). It was hypothesised that the alpha toxin had also been carried in vivo. It has been suggested that a common bacterial strain exists in nature, and that its toxigenicity and hence pathogenicity is governed by the presence of the specific converting phage (Eklund et al, 1974).

It is possible that the isolation technique did miss *C. botulinum* type C present in the samples. However, it is unlikely for several reasons that the organisms isolated in this study were not involved in the disease. Firstly, the organisms were all isolated from animals that either had dysautonomia at the time of sampling, or had previously had dysautonomia and recovered, or had recently been in contact with an animal with dysautonomia. No Group III organisms were isolated from an animal that had no history of exposure to dysautonomia. Secondly, the surface protein profiles of many of the isolates were very similar suggesting that the isolates were closely related. Several isolates were very similar despite geographical distances separating host animals from which they had been isolated and even despite species differences between host animals. Isolates with very similar/identical

surface protein profiles were isolated from a horse and hare both with dysautonomia; the hare was found on the same pasture as the horse. The majority of isolates in this study appear to be more closely related to each other than to the type strains. Although not all the samples from which these organisms were isolated were positive for BoNT/C, isolates with similar surface protein profiles were isolated from both BoNT/C-positive and BoNT/C-negative samples. All the isolates were also carrying the component I gene of the C2 toxin.

The isolation of these organisms from animals all connected in some way with dysautonomia, combined with similarities between isolates suggest that these organisms may play a role in dysautonomia. However, an improved method of isolation is essential to enable further characterisation of a larger number of these organisms that may be involved in the cause of equine grass sickness and related dysautonomias. The isolation rate of Group III organisms in this study has been very low: 27 out of 77 ileum samples and 26 out of 117 faecal samples had BoNT/C-producing organisms present as determined by toxin detection after enrichment but only six organisms were isolated from samples that had been shown to contain a BoNT/C-producing organism. The difficulties in isolating a toxigenic Group III organism have been previously discussed. Clearly this raises problems when, as in this case, trying to isolate and identify the aetiological agent of a disease putatively caused by a Group III organism.

On the basis of the results obtained from characterisation of the isolates to date, it is possible to hypothesise that animals at risk from dysautonomia are colonised by a *C. novyi* type A-like organism, and that these organisms either carry a bacteriophage encoding BoNT/C or they become infected with a phage carrying the BoNT/C gene enabling BoNT/C production. The bacteriophage could be introduced

into the resident population by another Group III organism; if the *C. novyi* type A was resident in the GI tract they would be less likely to be eliminated by a mucosal immune response. Further investigation is required to establish the carriage of Group III organisms in the equine GI tract and their distribution in the environment. Only six out of 72 control horses were found to carry toxigenic *C. botulinum* type C in their faeces (Chapter 3). However, the enrichment method used would not be able to detect the presence of non-toxigenic organisms or other Group III organisms. The use of PCR for the detection of Group III-specific 16s rRNA and toxin genes in GI samples, will enable the determination of the rate of carriage of both Group III organisms and specific species in the equine population.

The involvement of other Group III toxins in dysautonomia requires investigation. It has already been hypothesised that the C2 toxin could potentially cause some of the ultrastructural changes observed in neurons in equine grass sickness, but the role of the *C. novyi* alpha toxin has previously not been considered. The alpha toxin is a large clostridial cytotoxin (LCT) with lethal and necrotising activity; its role in the pathogenesis of gas gangrene is well established. However, the effects of the toxin when produced in the GI tract are not known. Novyi alpha toxin causes retraction and rounding in cultured endothelial cells. This characteristic cytopathic effect is associated with disintegration of microfilaments and redistribution of F-actin and vinculin (Oksche, Nakov and Habermann, 1992). This is due to modification of the Rho protein. Rho is involved in regulating the steady state of polymerisation of F actin (Ridley and Hall, 1992). Therefore, the alpha toxin, like the C2 and C3 botulinum toxins, causes microfilament disruption. In vitro, the morphological changes induced by the *C. novyi* alpha toxin on cultured cells are indistinguishable from the changes induced by the C2 toxin. The alpha toxin also shares the same intracellular target protein as the C3 toxin. However, the novyi alpha toxin has a

different mode of action to the C2 and C3 toxins. Three of the 16 isolates were shown to be carrying the alpha toxin gene, other isolates may have been carrying the gene in vivo. Further investigation is required to establish whether this toxin could also have a role in the aetiology of equine grass sickness.

Chapter Five

Systemic immune response to *C. botulinum* type C

5.1 Results

5.1.1 Detection of IgG in serum to *C. novyi* type A and BoNT/C

A wide range of IgG levels to *C. novyi* type A surface antigens and to BoNT/C were detected in horses that had grass sickness and those that did not have the disease (Table 5.1). The antibody levels to these antigens were compared between six categories of horses - horses with CGS, SGS and AGS, contacts, high risk and controls - as illustrated in Table 5.1. The IgG levels to BoNT/C and the surface antigens were also compared between different groupings of these horses (Fig. 5.1, 5.2 and 5.3). Horses with grass sickness were found to have a significantly lower level of serum IgG to *C. novyi* type A surface antigens ($p=0.01$) and to BoNT/C ($p<0.001$), compared to horses that did not have the disease (Fig. 5.1a and 5.1b).

The horses that did not have grass sickness were divided into three groups – 'controls', 'contacts' and 'high risk'. There was a significantly lower level of IgG to BoNT/C in the serum of horses with grass sickness, compared to horses that had been in contact with cases of the disease ($p<0.01$), and horses grazing high risk pasture ($p<0.001$) (Fig. 5.2a). There was also a significantly lower level of IgG to *C. novyi* type A surface antigens in the serum of horses with grass sickness ($p<0.05$), compared to horses in contact with cases of the disease and those grazing high-risk pasture (Fig. 5.2b). Horses with grass sickness had a lower mean level of serum

IgG to BoNT/C and *C. novyi* type A, compared to the control horses, but these were not significant differences.

Table 5.1: IgG to *C. novyi* type A surface antigens and to BoNT/C in equine serum.

Specific antibody	Statistics	Antibody level (OD_{405}nm)					
		CGS (n= 31)	**SGS (n=21)**	**AGS (n=28)**	**Controls (n=36)**	**Contact (n=60)**	**High risk (n=46)**
IgG to surface antigens	**Mean**	0.75	0.75	0.72	0.80	0.89	0.89
	SD	0.31	0.41	0.38	0.36	0.37	0.40
	Range	0.36-1.44	0.2-1.63	0.17-1.94	0.16-1.82	0.31-2.20	0.26-2.06
IgG to BoNT/C	**Mean**	0.17	0.22	0.18	0.24	0.30	0.36
	SD	0.19	0.17	0.15	0.19	0.34	0.30
	Range	0.04-0.62	0.02-0.63	0.03-0.66	0.01-0.94	0.02-2.44	0.07-1.23

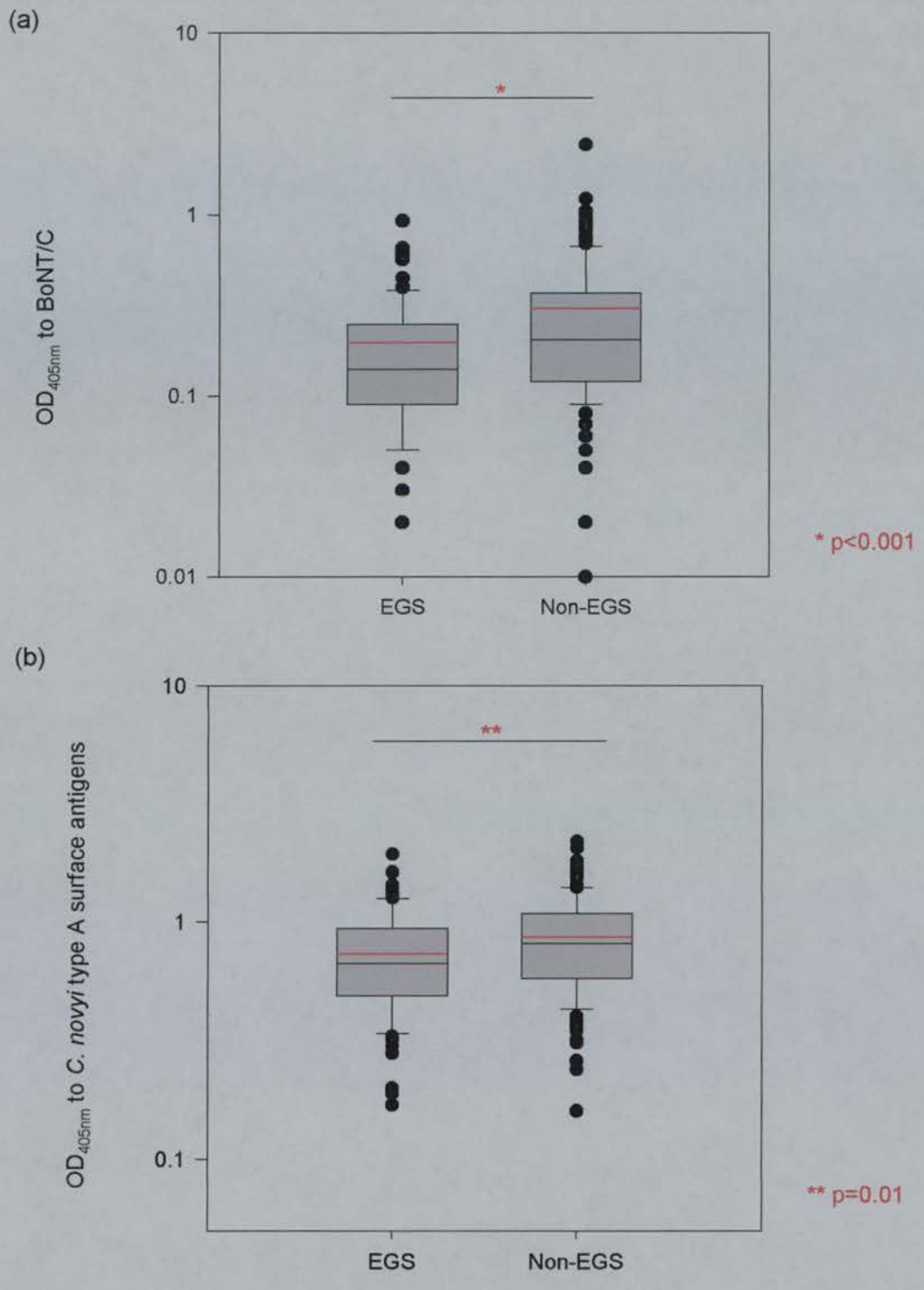


Figure 5.1: (a) IgG to BoNT/C and (b) IgG to *C. novyi* type A surface antigens in the serum of horses with and without equine grass sickness (EGS). The upper line of the box represents the 75th percentile, the lower line of the box the 25th percentile, and the middle black line in the box the median value for the distribution. The upper and lower error bars are the 90th and 10th percentiles, respectively. Circles represent outlying values. The red line is the mean value for the distribution.

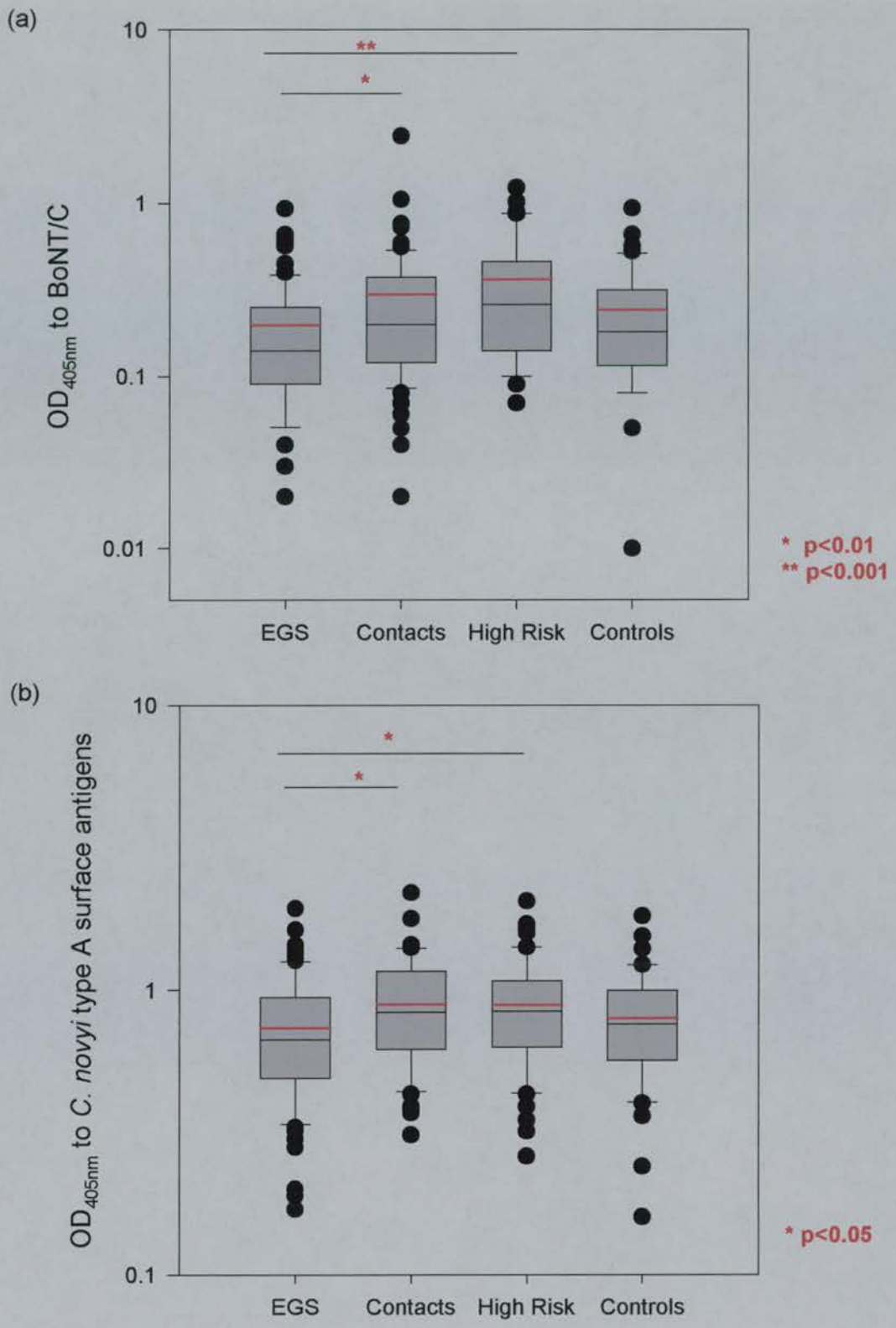


Figure 5.2: (a) IgG to BoNT/C and (b) IgG to *C. novyi* type A surface antigens in the serum of horses with EGS, horses in contact with EGS, horses grazing high risk pasture and controls not known to be in contact with EGS. The boxplot is as described for Figure 5.1.

The control horses consisted of 17 healthy horses, and 19 clinical cases, 10 of which had gastrointestinal (GI) associated disease. There was a higher mean IgG level to both BoNT/C and *C. novyi* type A surface antigens in the serum of horses with GI associated disease, compared to either the healthy controls or the non-GI clinical cases (Table 5.2). However there were no significant differences in serum IgG to either BoNT/C or *C. novyi* type A between healthy horses, GI cases and non-GI cases. There were no significant differences between IgG to BoNT/C or to *C. novyi* type A between horses in contact with a case of grass sickness, those grazing land with a high risk of grass sickness or the control horses.

Table 5.2: IgG to *C. novyi* type A surface antigens and to BoNT/C in the serum of the control horses.

		Antibody levels (OD_{405}nm)		
Specific antibody	**Statistics**	**Healthy controls (n=17)**	**GI clinical cases (n=10)**	**Non-GI clinical cases (n=9)**
IgG to *C. novyi* type A	**Mean**	0.77	0.84	0.80
	SD	0.31	0.36	0.47
	Range	0.36-1.55	0.24-1.40	0.16-1.82
IgG to BoNT/C	**Mean**	0.24	0.3	0.19
	SD	0.14	0.26	0.2
	Range	0.09-0.57	0.05-0.94	0.01-0.66

There was a significantly higher level of serum IgG to BoNT/C in the horses without EGS, compared to horses with chronic grass sickness ($p<0.01$) and acute grass sickness ($p<0.01$). Although there was a higher mean level of IgG to BoNT/C in horses without EGS compared to those with subacute grass sickness, this was not a significant difference. If horses with chronic and subacute grass sickness are grouped together (CGS/SGS) then there is a significantly lower level of IgG to BoNT/C in these horses compared to horses without EGS ($p<0.01$) (Fig. 5.3a).

There was a significantly higher level of serum IgG to BoNT/C in horses that were in contact with grass sickness, compared to those with CGS ($p<0.05$), CGS/SGS ($p<0.05$) and AGS ($p<0.01$). There was also a higher level of serum IgG to BoNT/C in horses that were grazing high risk property, compared to horses with CGS ($p=0.001$), CGS/SGS ($p=0.001$) and AGS ($p<0.005$).

There was a significantly higher level of serum IgG to the surface antigens in horses without EGS compared to those with CGS/SGS ($p<0.05$), or AGS ($p<0.05$) (Fig. 5.3b). There were no significant differences between horses without grass sickness and those with CGS or SGS, if the two groups were considered separately.

There was a significantly higher level of serum IgG to the surface antigens in the horses in the contact group compared to horses with CGS/SGS or AGS ($p<0.05$). Although the mean level of IgG to the surface antigens was higher in the horses grazing high-risk pasture and in the controls, than horses with AGS or CGS and SGS, these were not significant differences.

There were no significant differences in serum IgG levels to BoNT/C or surface antigens between horses with CGS, SGS or AGS.

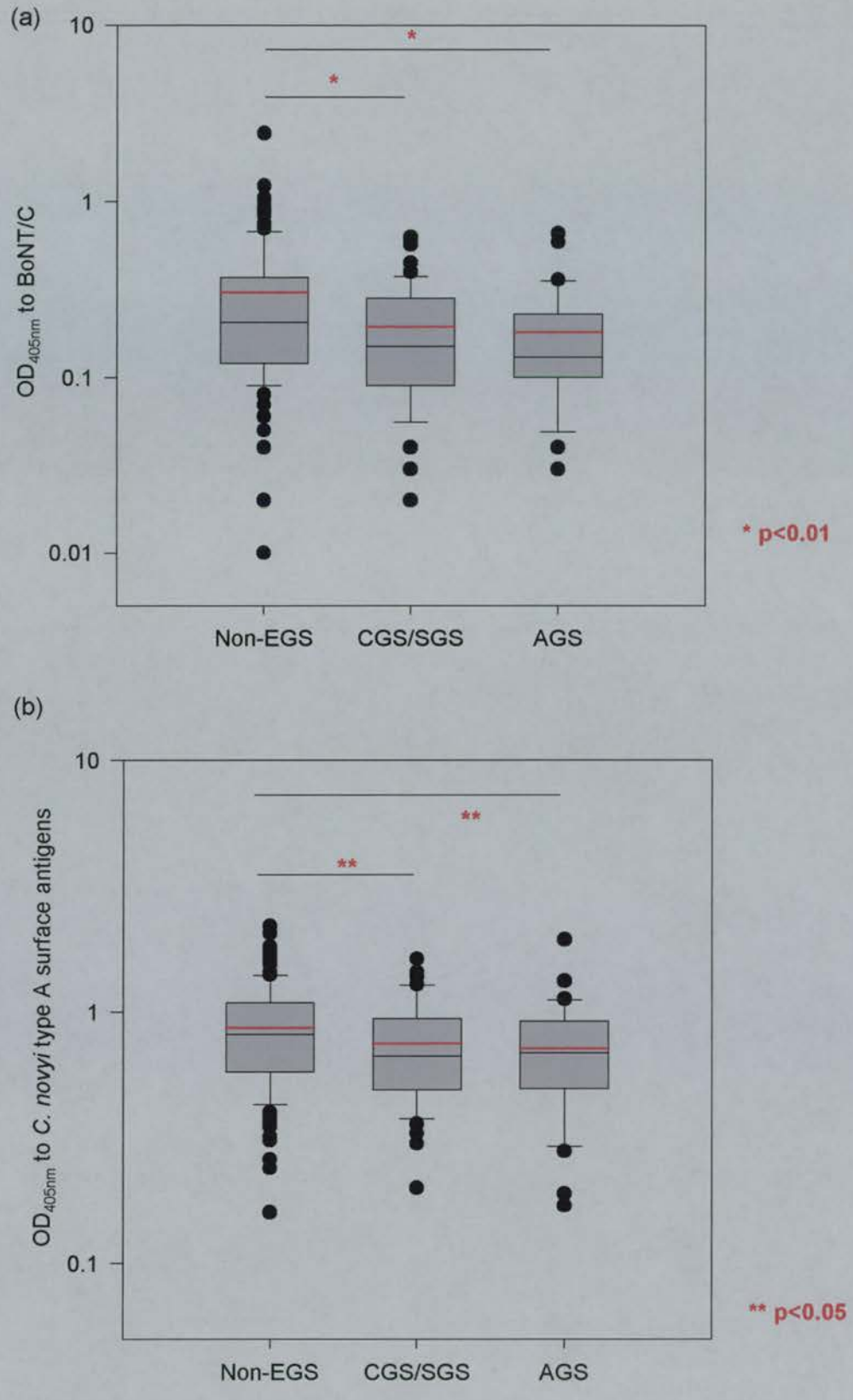


Figure 5.3: (a) IgG to BoNT/C and (b) IgG to *C. novyi* type A surface antigens in the serum of horses with CGS/SGS, AGS and horses without equine grass sickness (EGS). Boxplot is as described for Fig. 5.1.

5.1.2 IgG to *C. novyi* type A and BoNT/C in consecutive serum samples from horses with CGS

Fourteen horses with chronic grass sickness were sampled more than once during the course of the disease: seven horses were sampled twice (Table 5.3), and seven horses were sampled more than twice (Fig. 5.4). Ten of these 14 horses showed an increase in OD both to *C. novyi* type A surface antigens and to BoNT/C in at least one sample over the course of the disease; three horses showed an increase in OD to the surface antigens only; one horse showed an increase to BoNT/C only (Fig. 4a-g and Table 3). In some cases the increase in OD was very small.

Fluctuations in antibody levels were observed over the course of the disease (Fig. 5.4). These fluctuations were not always the same for both the surface antigens and BoNT/C. Recovery of a horse from CGS was not always associated with an increase in specific antibody levels to the surface antigens or the toxin (Fig. 5.4). In all cases the OD for IgG to BoNT/C was much lower than the OD for IgG to *C. novyi* type A. However, this reflects differences in the respective ELISA assays, and not necessarily differences in levels *in vivo*.

Table 5.3: Serum IgG to *C. novyi* type A and BoNT/C in horses with chronic grass sickness that were sampled twice during the course of the disease.

Horse ID	Day sampled	IgG to surface antigens (OD_{405nm})	IgG to BoNT/C (OD_{405nm})	Duration of disease (days)	Outcome
LH335	6	0.74	0.05	9	Euthanasia
	8	0.80	0.03		
LH 338	2	0.90	0.08	8	Euthanasia
	3	0.93	0.09		
LH174	3	0.39	0.06	35	Euthanasia
	9	0.46	0.07		
LH178	6	0.43	0.15	21	Euthanasia
	14	0.54	0.19		
LH236	38	0.56	0.12	47	Recovered
	47	0.55	0.16		
LH219	26	0.52	0.37	32	Recovered
	29	0.56	0.34		
LH227	11	0.84	0.13	26	Euthanasia
	26	1.05	0.20		

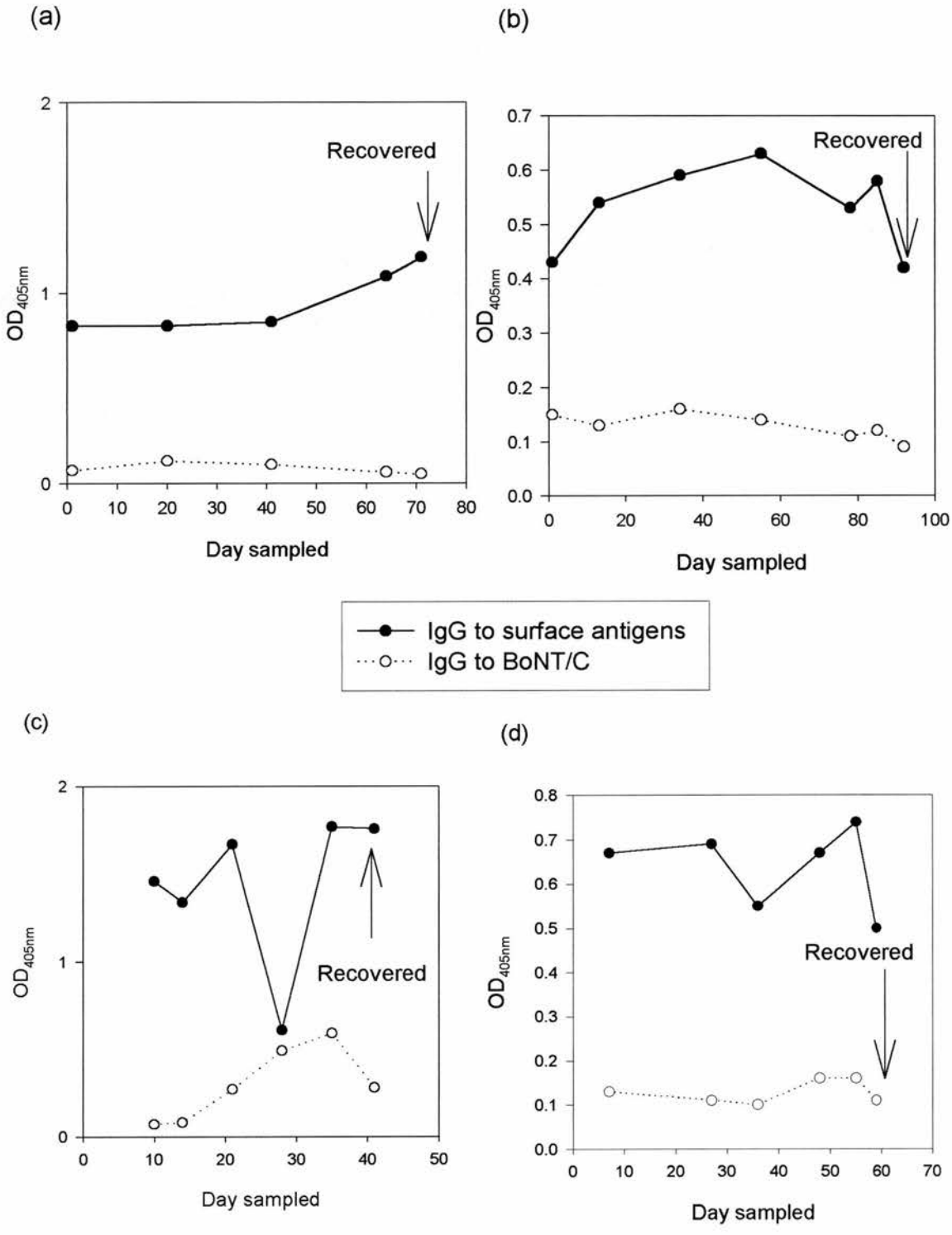
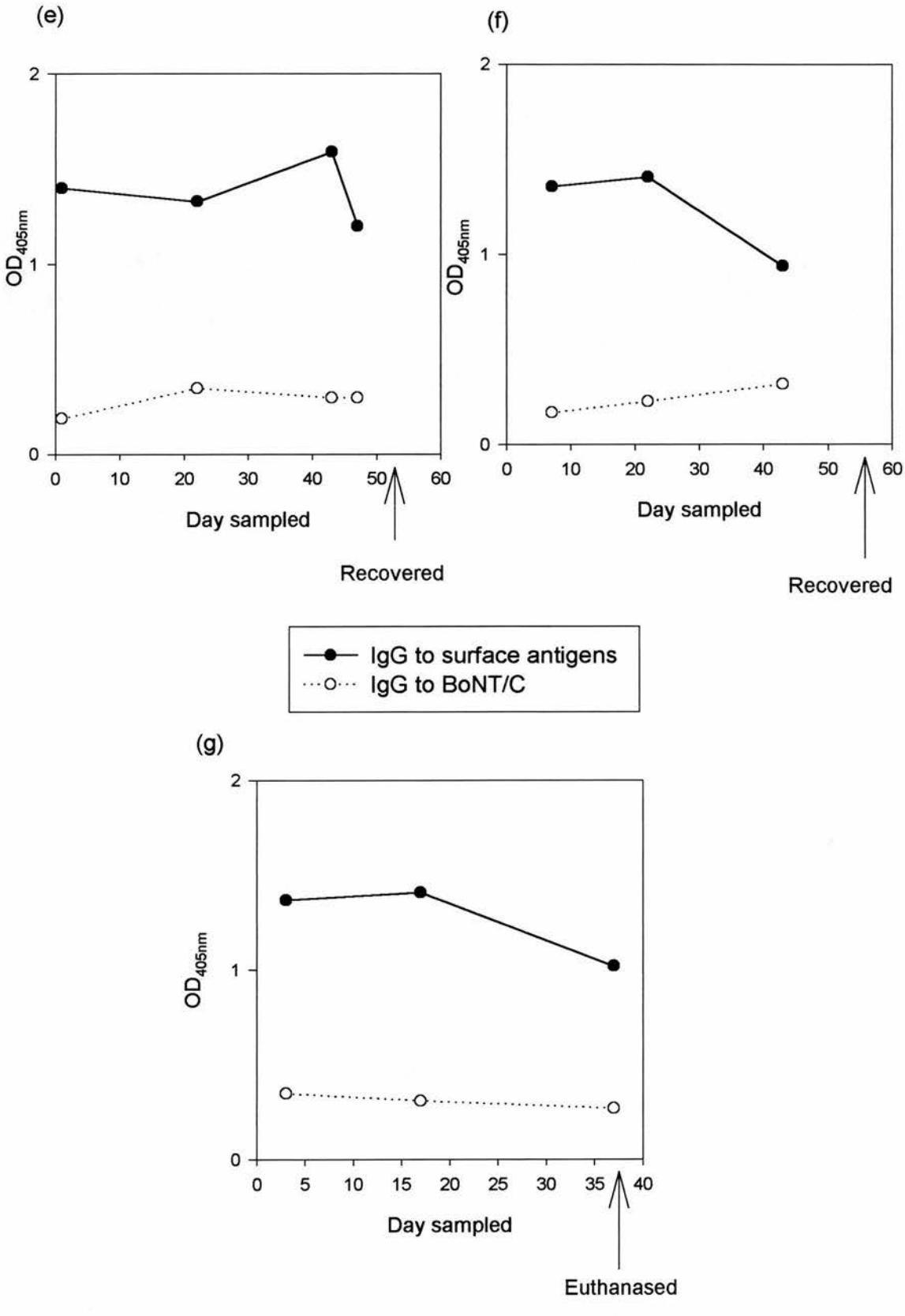


Figure 5.4: (a)-(g) Serum IgG to *C. novyi* type A surface antigens and BoNT/C in seven horses with chronic grass sickness sampled over the course of the disease. The arrows on each graph show the endpoint of disease for each horse, and the the outcome- either euthanasia or recovery.


(e)
(f)
2
1
0
OD_{405nm}
0 10 20 30 40 50 60
Day sampled
Recovered
IgG to surface antigens
IgG to BoNT/C
(g)
0 5 10 15 20 25 30 35 40
Euthanased

5.1.3 Comparison of specific IgG between horses with CGS that recovered and those that were euthanased

The levels of serum IgG to surface antigens and to BoNT/C were compared between horses that had recovered from chronic grass sickness and those that were euthanased. When more than one sample had been received from a horse, the OD values were averaged - Table 5.3 and Fig. 5.4 illustrate the range of antibody levels detected in these horses. There was a higher mean IgG level both to *C. novyi* type A surface antigens and to BoNT/C in the serum of horses that had recovered from CGS, compared to those that were euthanased (Table 5.4). However, these were not statistically significant differences.

Specific IgG levels, at both the start and the end of the disease, were compared between horses that recovered from CGS and those that were euthanased. The horses that recovered from CGS had a higher mean level of IgG to the surface antigens, at the start of the disease, compared to the horses that did not recover (Table 5.5a). However, the horses that did not recover had a higher mean level of IgG to both surface antigens and BoNT/C, at the end of the disease (prior to euthanasia), than the horses that recovered (Table 5.5b). These results are not statistically significant.

Table 5.4: IgG to *C. novyi* type A surface antigens and to BoNT/C in the serum of horses with chronic grass sickness. A comparison between horses that were euthanased and those that recovered from the disease.

Horses	Statistics	Antibody levels OD_{405nm}	
		IgG to surface antigens	IgG to BoNT/C
CGS cases that recovered (n= 11)	**Mean**	0.84	0.18
	SD	0.35	0.10
	Range	0.53-1.44	0.07-0.36
CGS cases that were euthanased (n=18)	**Mean**	0.69	0.14
	SD	0.27	0.11
	Range	0.36-1.27	0.04-0.45

Table 5.5: Comparison of IgG to surface antigens and to BoNT/C between horses that recovered from CGS and those that were euthanased, (a) specific IgG at beginning of the disease and (b) specific IgG levels at end of disease.

(a)

Horses with CGS	Antibody levels OD_{405nm}	
	Mean IgG to surface antigens at start of disease	Mean IgG to BoNT/C at start of disease
Recovered (n=3)	0.89	0.14
Euthanased (n=4)	0.79	0.14

(b)

Horses with CGS	Antibody levels OD_{405nm}	
	Mean IgG to surface antigens at end of disease	Mean IgG to BoNT/C at end of disease
Recovered (n=4)	0.67	0.10
Euthanased (n=4)	0.81	0.14

5.1.4 Longitudinal study of six healthy controls

Serum samples were collected from six healthy control horses at intervals over a 28-week period. The horses were stabled when samples were collected at weeks one, and four. The other samples were collected when the horses were grazing pasture. Grass sickness was not known to have occurred previously on this pasture. Two of the horses showed an increase in serum IgG to the surface antigens towards the end of the collection period: at week 19 for horse B3 (Fig. 5.5c) and at week 28 for horse B4 (Fig. 5.5d). Horse B4 also showed an increase in serum IgG to BoNT/C (Fig. 5.5d). The other four horses showed minor fluctuations in specific antibody levels over the course of the collection period (Fig. 5.5a, b, e and f).

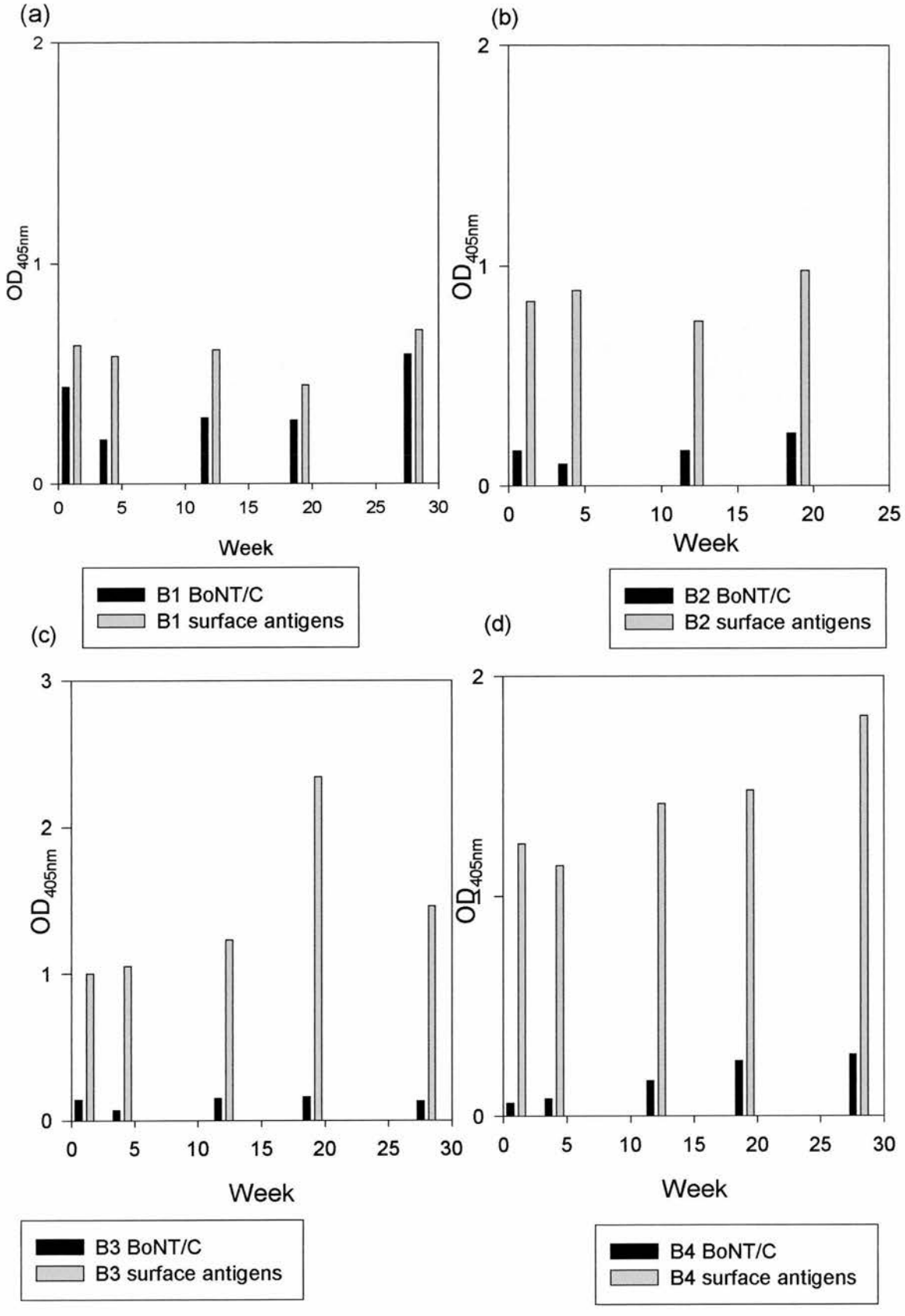
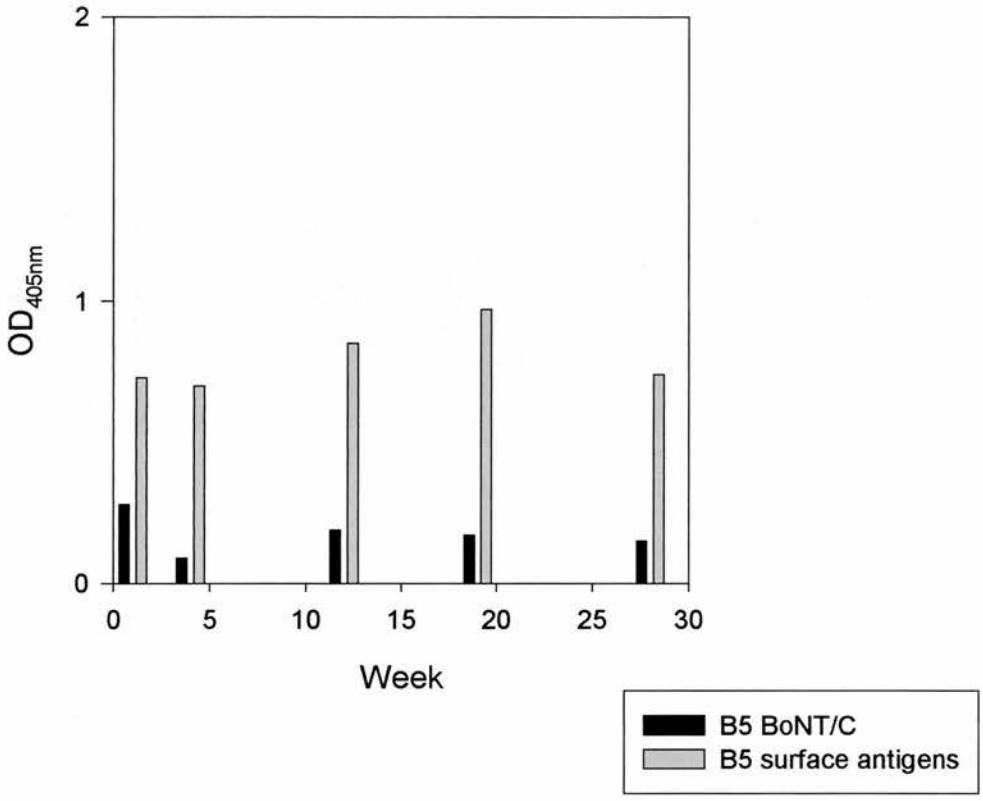
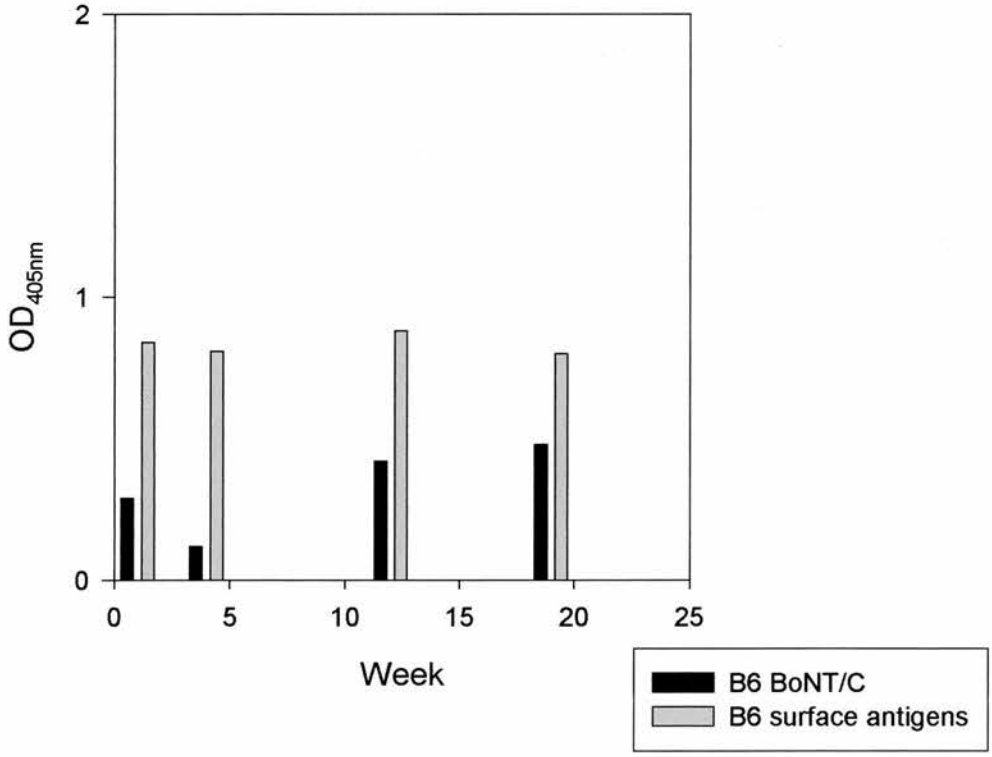


Figure 5.5: (a)-(f) IgG to *C. novyi* type A surface antigens and to BoNT/C in six control horses sampled over a period of 28 weeks.

(e)


2
1
0
OD405nm
0
5
10
15
20
25
30
Week
B5 BoNT/C
B5 surface antigens

(f)


2
1
0
OD405nm
0
5
10
15
20
25
Week
B6 BoNT/C
B6 surface antigens

5.2 Discussion

The surface antigens of *C. novyi* type A were used as a phenotypic marker *for C. botulinum* type C. It has been shown that the surface antigens of these organisms are serologically cross-reactive (Poxton, 1984). Bulk cultures were required for the extraction of surface antigens for use in the ELISA, and it was considered safer to grow up large volumes of *C. novyi* type A rather than neurotoxigenic *C. botulinum* type C, especially as there were unvaccinated personnel in the laboratory. For later work (Chapter 7), a non-neurotoxigenic *C. botulinum* type C strain was obtained for large-scale extraction of surface antigens. The surface antigens of this strain were also shown to be serologically cross-reactive with the surface antigens of *C. novyi* type A (Chapter 4.1.3).

Epidemiological evidence suggests that resistance can occur to grass sickness. There is a decreased incidence of the disease in older horses, in horses that have been in prior contact with cases of grass sickness, and horses that have been on a particular pasture for a longer length of time (Gilmour and Jolly, 1974; Doxey, et al, 1991b; Wood et al, 1998). This resistance may be in the form of an immune response to the aetiological agent. Some experimental ponies were found to be resistant to the toxic factor present in plasma from acute grass sickness horses that had been shown to cause neuronal chromatolysis when injected into other ponies (Doxey et al, 1997). The intraparotid injection of serum from a horse with chronic grass sickness resulted in an inflammatory reaction in the cranial cervical ganglion (Griffiths et al, 1994). It was thought that this may have been part of an immune-mediated response – antibodies may have developed to the neurotoxin (or components of the autonomic ganglia) in the chronically affected horse.

There was a significantly higher level of IgG both to the surface antigens and to BoNT/C in the serum of horses that had been in contact with grass sickness, compared to the horses that actually had the disease. This suggests that horses in contact with grass sickness have been exposed both to the surface antigens of a Group III organism and to BoNT/C and have made a systemic immune response. A case-control epidemiological study has shown that prior contact with a case of grass sickness is associated with a ten-fold decrease in the likelihood of the disease (Wood et al, 1998). It was hypothesised that this observed resistance occurring in horses in contact with the disease could be in the form of an immune response to the aetiological agent. The data from this study of specific systemic antibody levels would support this hypothesis.

There was also a significantly higher level of IgG both to the surface antigens and to BoNT/C in the serum of horses grazing land considered to be a high risk for equine grass sickness (grass sickness had occurred there frequently in the past), compared to horses with the disease. This data would suggest that these horses have also been exposed to subclinical levels of both the organism and the toxin, resulting in a systemic immune response. However, it is not known whether these specific serum antibody levels are protective in these horses. The control horses were not known to have been exposed to grass sickness and although they had lower mean specific IgG levels compared to the horses in contact with grass sickness or grazing pasture where the disease is endemic, these were not significant differences. Subclinical cases of grass sickness are thought to occur, but have not been proven (Doxey et al, 1995a; Milne, 1997).

Detection of lower specific antibody levels to the surface antigens and BoNT/C in horses with grass sickness could also suggest that these horses may be more

susceptible to the disease due to an inadequate immune response to *C. botulinum* type C. However, although the control horses had a higher mean IgG level to these antigens, they were not significantly different from the horses with grass sickness.

There were no significant differences in antibody levels between the three categories of grass sickness. It has been hypothesised that a higher level of specific IgG can be detected to these antigens in the serum of horses with chronic grass sickness, compared to horses with the acute form of the disease, due to seroconversion (J.K. Miller, personal communication). However, the data from this study does not support this theory. An important consideration may be the time-point in the course of the disease at which the sample is collected – horses surviving for longer may have higher antibody levels. Chronic grass sickness can last from seven days to several weeks and even months. In this study, 14 horses with chronic grass sickness were sampled more than once. When there was more than one sample for a horse, the results were averaged for the purpose of comparing the IgG levels between the different categories of horses.

All 14 of the horses with chronic grass sickness that were sampled more than once, showed an increase in OD to either the surface antigens, BoNT/C or both at least once during the course of the disease. However, it is doubtful whether this could be interpreted as seroconversion as the majority of observed increases were very small changes in OD and were often transient. Decreases in OD to these antigens were also observed. The fluctuations in OD levels to these antigens over the course of the disease could be due to the in vivo neutralisation of the organism and toxin, resulting in a reduction in the level of IgG available for detection by the ELISA. This may also explain why horses with grass sickness have a significantly lower level of IgG to these antigens compared to horses without the disease.

If seroconversion occurred during chronic grass sickness, it would seem plausible that horses with a higher serum IgG level to the surface antigens and BoNT/C would have a better prognosis and be more likely to recover. Horses that recovered from chronic grass sickness did have higher mean antibody levels to BoNT/C and surface antigens than the horses with chronic grass sickness that were euthanased. However, lower IgG levels to both the surface antigens and toxin were detected at the end of the disease course in horses that recovered, compared to those that were euthanased. These results are based on only eight horses and are not statistically significant. If systemic antibodies play a role in the prognosis of disease, it might be expected that horses with higher specific antibody levels at the start of the disease, would be more likely to recover. Based on IgG levels from only seven horses, sampled within three days of onset of clinical symptoms, it was found that horses that recovered from CGS had higher IgG levels to the surface antigens, but not to BoNT/C, compared to horses that were eventually euthanased. However, serum samples collected even on the first day of clinical signs are not 'pre-exposure' samples, as the horse may have been exposed to the organism and toxin for some time before the onset of clinical symptoms. Again, these results were not statistically significant.

Antitoxin immunity is not generally thought to develop in humans with food-borne botulism; it was considered that the lethal dose of botulinum toxin was less than the antigenic dose (Griffin et al, 1997). However, endogenous antibody production to botulinum toxin has been demonstrated in a case of adult intestinal colonisation botulism (Griffin et al, 1997); it was not known whether this contributed to the patient's recovery. Cattle (Jean et al, 1995) and horses (Whitlock and Buckley, 1997) recovering from type C botulism have been shown to develop protective

antibody; antibody to botulinum toxin was also detected in healthy cattle in areas where botulism was considered endemic (Gregory et al, 1996).

Fluctuation in antibody levels to the surface antigens and BoNT/C were also observed in six healthy control horses, sampled at intervals over a 28-week period. All of the six horses showed an increase in OD to either the surface antigens, BoNT/C or both over the collection period. The six horses all had detectable antibodies to these specific antigens, and the antibody levels differed between the six horses, despite grazing the same pasture. An increase in OD may be evidence of recent exposure to the organism and toxin. The collection period ran from February to September. There is some evidence of an increase in IgG to the surface antigens and BoNT/C, in some of the horses, between samples collected in May and September; this may reflect exposure to *C. botulinum* type C in the spring/summer months. However, more samples are required to draw conclusions with respect to seasonal exposure and fluctuations in immunity. It would be particularly interesting to study fluctuations in antibody levels in horses grazing pasture where grass sickness is considered to be endemic.

All horses in this study had detectable IgG to both the surface antigens and BoNT/C, suggesting that they had all been exposed to *C. botulinum* type C and the toxin. The higher mean specific antibody levels detected in horses exposed to grass sickness, either through co-grazing with a case or grazing pasture where the disease has frequently occurred, may reflect more recent exposure to *C. botulinum* type C. Although the systemic immune response may protect horses in contact with grass sickness from developing the disease, it does not appear to play a significant part in natural recovery from the disease. The mucosal immune system may prove to be more important in both the prevention of, and recovery from, grass sickness.

Chapter Six

Mucosal immune response to *C. botulinum* type C in the equine gastrointestinal tract

6.1 Results

6.1.1 Optimisation of ELISA to detect IgA in GI contents to BoNT/C and *C. novyi* type A surface antigens.

ELISA to detect IgA to *C. novyi* type A

Preliminary experiments were carried out to establish the appropriate dilution range for both the samples and primary detecting antibody for an ELISA to detect IgA in equine GI contents. Initial experiments used plates coated with *C. novyi* type A, as this antigen was readily available. A sample of equine ileum contents and a faecal sample were diluted twofold between 1 in 2 and 1 in 128; the primary detecting antibody, anti-equine IgA (mouse monoclonal) was used at a dilution of 1 in 100 and 1 in 1000. The results of these initial experiments demonstrated that higher OD levels were obtained when the primary antibody was used at a dilution of 1 in 100, for both the ileum contents (Fig. 6.1a) and the faecal sample (Fig. 6.1b). A linear relationship was observed between the 1 in 2 and 1 in 8 dilutions of ileum contents (Fig. 6.1a). The 1 in 4 dilution of ileum contents appeared to be a suitable dilution for assaying further samples. The dilution curve for the faecal sample was less consistent than that for the ileum contents (Fig. 6.1b). However, the results suggested that a dilution between 1 in 4 and 1 in 16 would be appropriate.

Further assays were carried out with twofold dilutions of the primary detecting antibody, anti-equine IgA, between 1 in 100 and 1 in 1600, against twofold dilutions

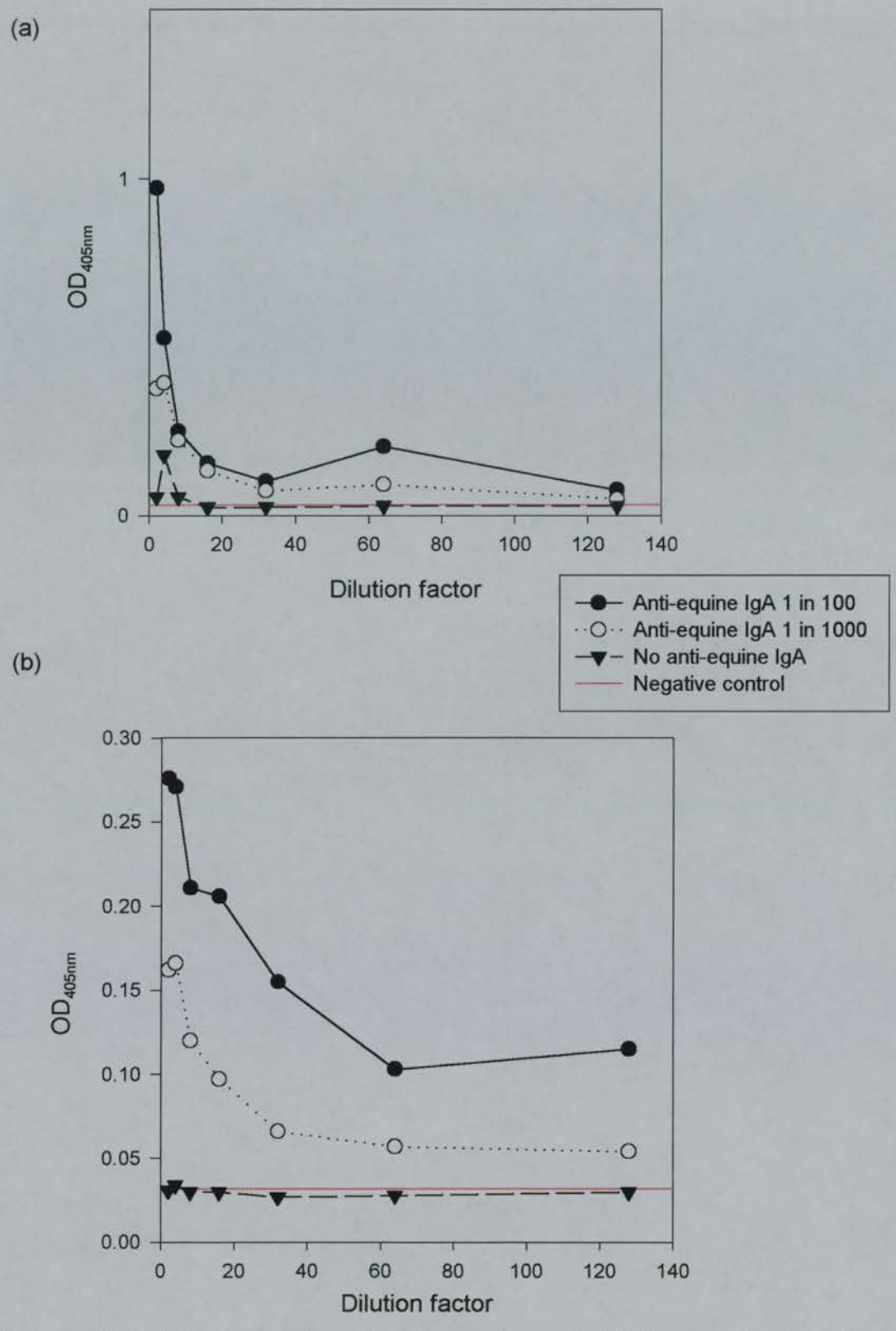


Figure 6.1: IgA to *C. novyi* type A surface antigens in (a) ileum contents and (b) faeces. Each sample was diluted twofold, from 1 in 2 to 1 in 128, with the detecting anti-equine IgA monoclonal antibody used at dilutions of 1 in 100 and 1 in 1000. Results are also shown for each sample when no detecting anti-equine IgA was added. These are representative results from single experiments.

of the ileum contents between 1 in 2 and 1 in 8. The 1 in 200 dilution of the anti-equine IgA gave the highest OD readings at both 1 in 2 and 1 in 4 dilutions of the ileum contents, and the relationship between the three dilution points looked the most linear compared to the other anti-equine IgA dilutions (Fig. 6.2). The 1 in 1600 dilution of anti-equine IgA in this experiment gave almost identical OD readings at the 1 in 4 sample dilution as the 1 in 200 dilution of this primary detecting antibody (Fig. 6.2). However, the OD reading for the 1 in 8 sample dilution was almost the same as the 1 in 4 dilution, indicating the results for the 1 in 1600 dilution of detecting antibody did not follow a linear relationship between sample dilutions 1 in 2 and 1 in 8 (Fig. 6.2). Further assays suggested that the high OD readings observed for the 1 in 1600 dilution in this experiment were anomalous. Assays using different faecal samples demonstrated that the 1 in 4 dilution of faeces and the 1 in 200 dilution of anti-equine IgA would be a suitable dilution for this ELISA (Fig. 6.3a and 6.3b). The 1 in 100 dilution of anti-equine IgA did give slightly higher OD readings, but was less economical to use at this dilution.

ELISA to detect IgA to BoNT/C

Sample and anti-equine IgA monoclonal dilutions were also investigated for the ELISA to detect IgA to BoNT/C. Doubling dilutions of a sample of ileum contents from 1 in 2 to 1 in 32 were added to BoNT/C coated plates. The primary detecting antibody, anti-equine IgA monoclonal was used at twofold dilutions between 1 in 100 and 1 in 800. The results (Fig. 6.4) indicated that the 1 in 4 dilution of sample, with a 1 in 200 dilution of anti-equine IgA monoclonal, were appropriate dilutions for use in the screening ELISA for IgA to BoNT/C as well as to the surface antigens.

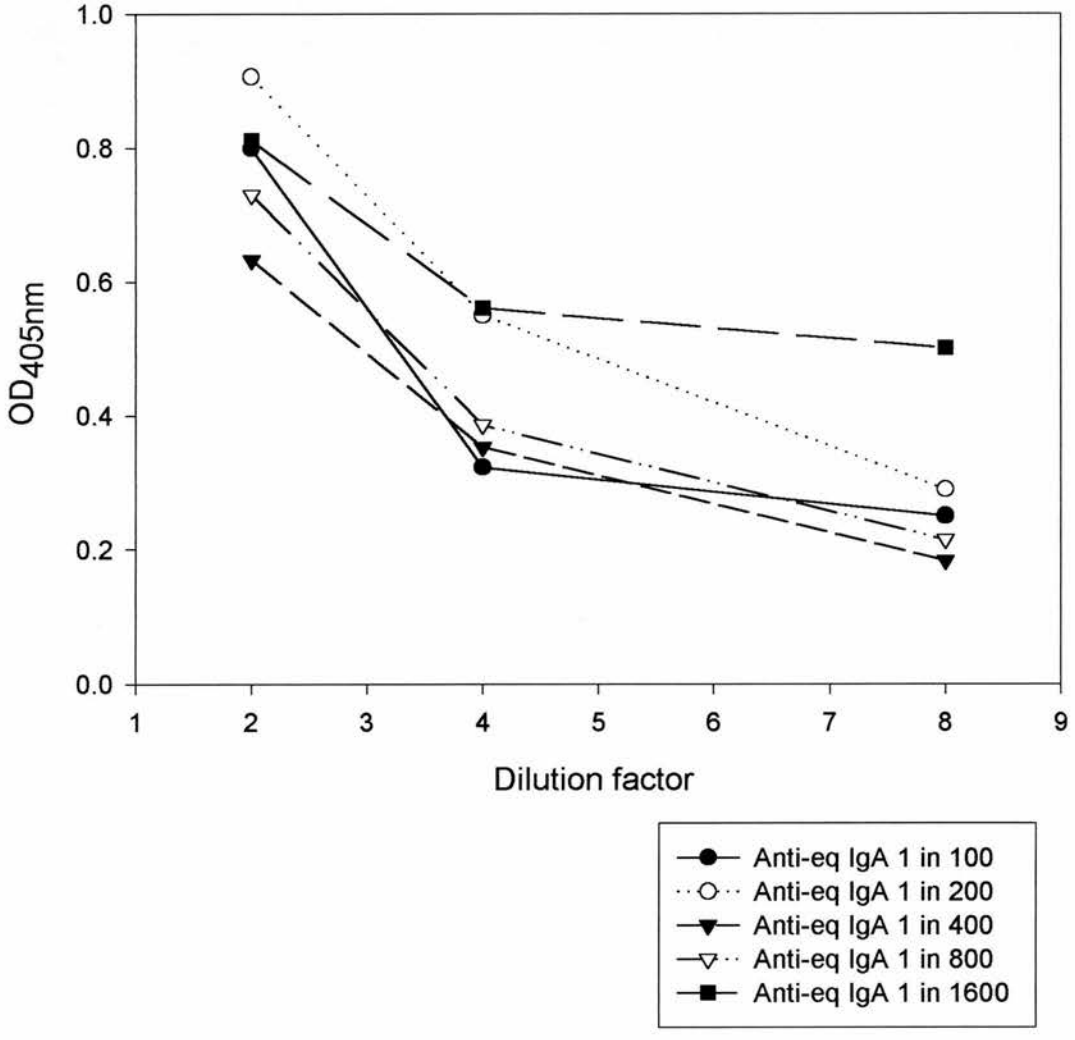
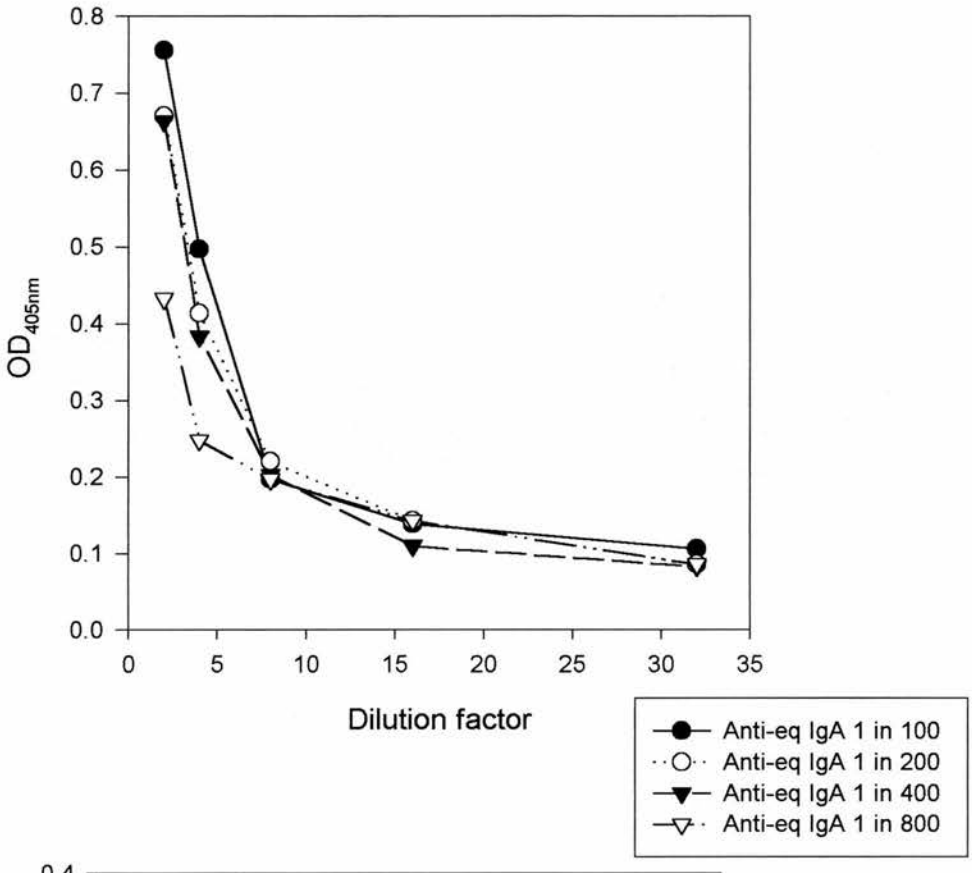


Figure 6.2: IgA to *C. novyi* type A surface antigens in ileum contents. Ileum contents were diluted twofold, from 1 in 2 to 1 in 8, with the detecting anti-equine IgA monoclonal antibody diluted twofold, from 1 in 100 to 1 in 1600. These are representative results from a single experiment.

(a)



(b)

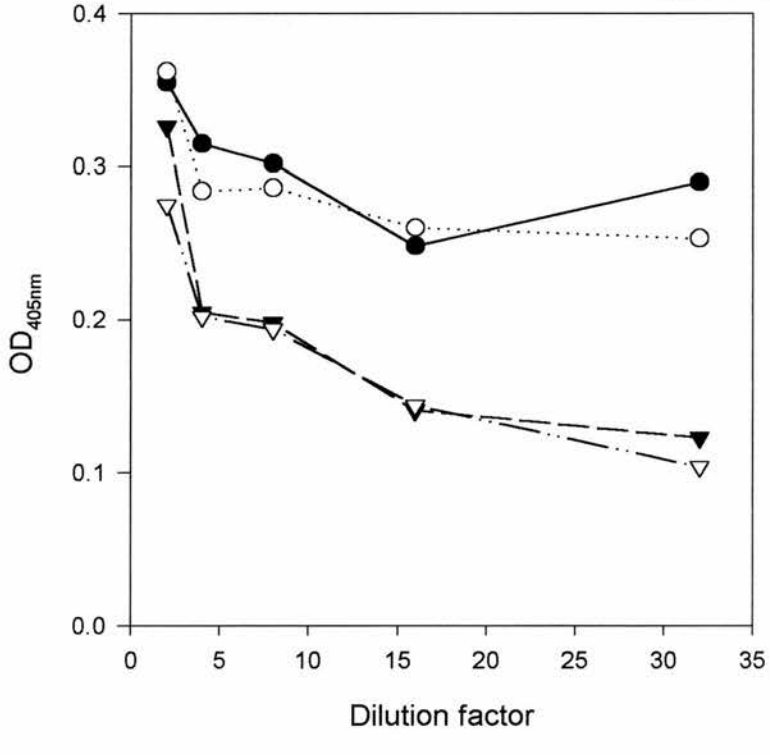


Figure 6.3(a) and (b): IgA to *C. novyi* type A surface antigens in two different faecal samples. Faecal samples were diluted twofold between 1 in 2 and 1 in 32, with the detecting anti-equine IgA monoclonal antibody diluted twofold between 1 in 100 and 1 in 800.

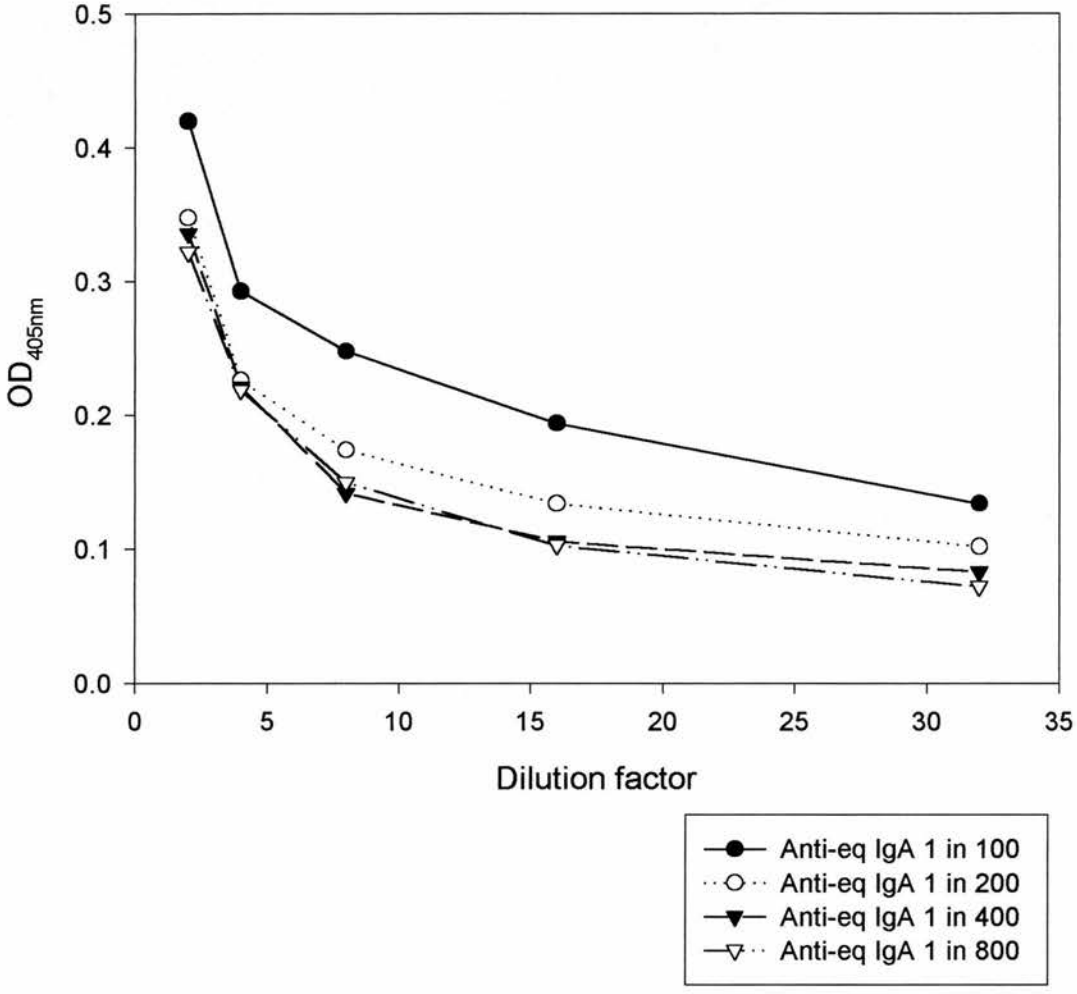


Figure 6.4: IgA to BoNT/C in ileum contents. Ileum contents were diluted twofold, from 1 in 2 to 1 in 32, with twofold dilutions of detecting anti-equine IgA monoclonal antibody, from 1 in 100 to 1 in 800.

Controls

Initially, samples were added to uncoated wells to control for non-specific binding to the plate. In preliminary assays, each sample received no primary detecting antibody to control for non-specific binding of the conjugate to the sample. However, as there was no significant binding of samples, or other reagents to uncoated wells, and no significant binding of conjugate directly to sample, in the absence of a primary detecting antibody, these controls were not incorporated into the final screening assay. Inclusion of the control for the non-specific binding of the conjugate to the sample halved the number of samples that could be assayed on each plate. Sufficient samples were assayed initially, with this control, to indicate that there was little variation between samples, and this control could be confidently left out of future screening assays using these reagents. The controls that were included for the screening assay were a positive and negative control. The positive control was a sample that had been identified in initial experiments as having a good OD reading in the ELISA. This control was then included on each plate to enable standardisation of OD readings between assays. The negative control received only antiserum/ conjugate diluent at the sample incubation stage, but received all other reagents.

6.1.2 Detection of specific IgA to *C. novyi* type A surface antigens and BoNT/C in faeces and ileum contents

Horses with SGS had detectable IgA to BoNT/C in the highest percentage of both ileum and faecal samples compared to the other groups of horses with and without the disease. Ileum samples had detectable IgA to BoNT/C from 83% (5/6) of horses with SGS, compared to ileum samples from 66.7% (8/12) of horses without grass sickness, 60% (3/5) of horses with CGS and 33.3% (3/9) of horses with AGS (Fig.

6.5a). IgA to BoNT/C was detected in all of the faecal samples tested for horses with SGS (4/4), compared to faecal samples from 80% (12/15) of horses with CGS, 57.1% (4/7) of horses with AGS and 52.1% (25/48) horses without grass sickness (Fig. 6.5a).

IgA to *C. novyi* type A surface antigens was detected in ileum samples from 80% (4/5) of horses with CGS, compared to ileum samples from 66.7% (4/6) of horses with SGS, 33.3% (4/12) of horses without grass sickness and 11.1% (1/9) of horses with AGS (Fig. 6.5b). IgA to the surface antigens was detected in faecal samples from 75% (3/4) of horses with SGS, compared to faecal samples from 46.7% (7/15) of horses with CGS, 42.9% (3/7) of horses with AGS and 43.8% (21/48) of horses without grass sickness (Fig. 6.5b).

A higher percentage of horses with grass sickness had detectable IgA both to BoNT/C and to the surface antigens in the ileum and/or faeces compared to horses without grass sickness. 71% (22/31) horses with grass sickness had detectable IgA to BoNT/C in the ileum and/or the faeces compared to 56.3% (27/48) horses without the disease. 58.1% (18/31) horses with grass sickness have IgA to *C. novyi* type A surface antigens in the ileum and/or the faeces, compared to 43.8% (21/48) horses without the disease.

Horse with grass sickness had a statistically significant higher level ($p<0.02$) of IgA to BoNT/C in their faeces compared to horses without grass sickness (Fig. 6.6). Horses with SGS had the highest mean level of IgA to BoNT/C in their faeces, followed by horses with AGS, then horses with CGS; horses without grass sickness had the lowest mean IgA level to BoNT/C (Table 6.1). The difference in IgA levels to BoNT/C between horses with SGS and CGS was statistically significant ($p<0.05$), as

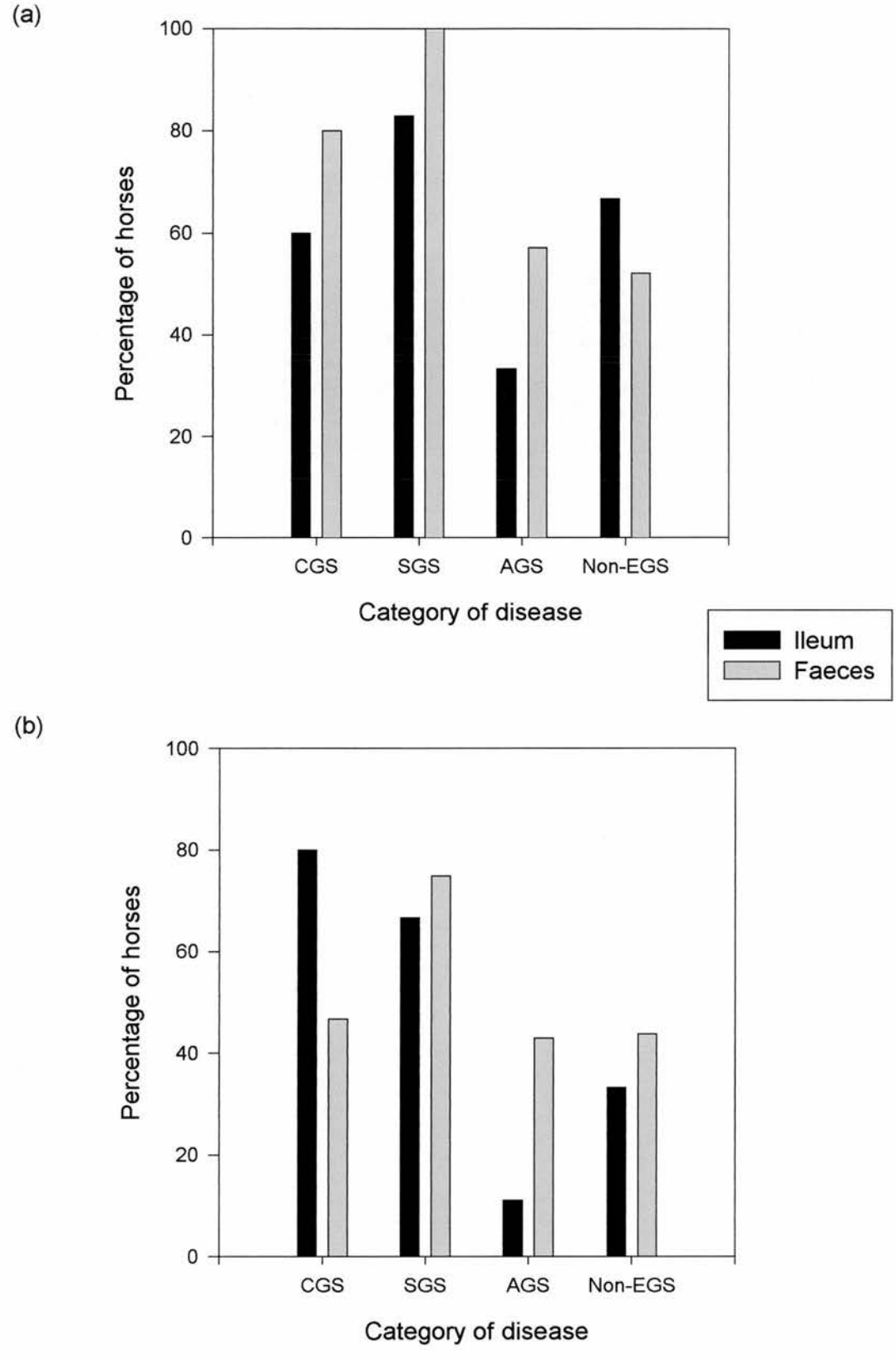


Figure 6.5: Percentage of horses with detectable IgA to (a) BoNT/C and (b) to *C. novyi* type A surface antigens in the ileum and faeces of horses with CGS, SGS, AGS and horses without grass sickness (non-EGS).

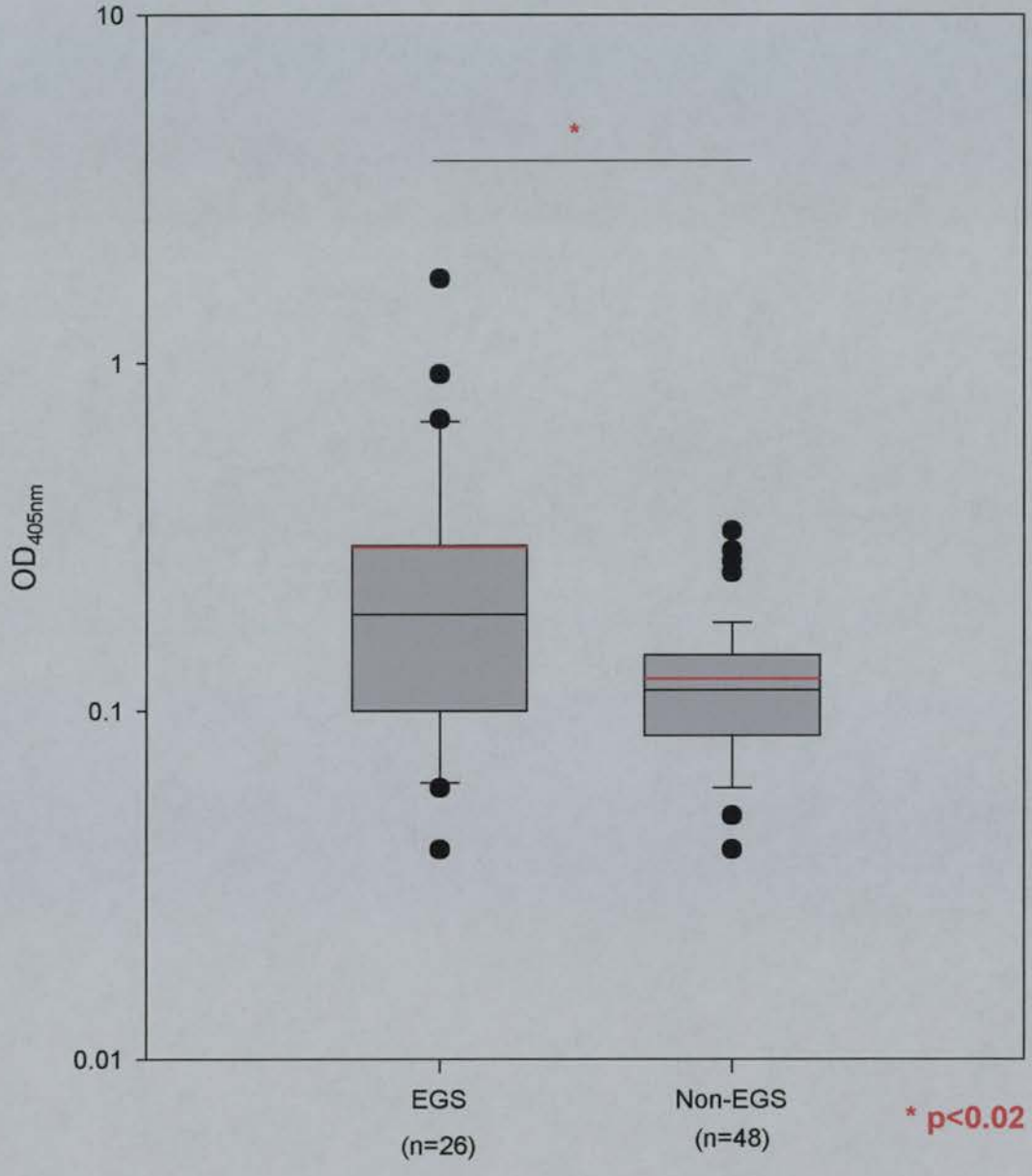


Figure 6.6: IgA to BoNT/C in the faeces of horses with EGS and horses without EGS. The upper line of the box represents the 75th percentile, the lower line of the box the 25th percentile, and the middle black line in the box the median value for the distribution. The upper and lower error bars are the 90th and 10th percentiles respectively. Circles represent outlying values. The red line is the mean value for the distribution.

was the difference between horses with SGS and horses without grass sickness ($p<0.01$).

Horses with SGS also had the highest mean level of IgA to *C. novyi* type A surface antigens in their faeces (Table 6.1). The level of IgA to the surface antigens was significantly higher in horses with SGS compared to horses without grass sickness ($p<0.05$); this was the only statistically significant difference between these groups of horses with respect to IgA to the surface antigens.

Table 6.1: IgA to *C. novyi* type A and BoNT/C in faeces of horses with CGS, SGS, AGS and horses without grass sickness (non-EGS).

Statistics		Antibody levels OD_{405nm}			
		CGS (n=15)	**SGS (n=4)**	**AGS (n=7)**	**Non-EGS (48)**
IgA to BoNT/C	**Mean**	0.17[1]	0.88[2]	0.24	0.12
	SD	0.11	0.67	0.19	0.06
	Range	0.04-0.45	0.15-1.75	0.06-0.58	0.04-0.33
IgA to surface antigens	**Mean**	0.11	0.43[3]	0.11	0.10
	SD	0.07	0.32	0.09	0.06
	Range	0.04-0.29	0.05-0.84	0.02-0.26	0.02-0.29

1. Statistically significant difference between horses with CGS and SGS ($p<0.05$)
2. Statistically significant difference between horses with SGS and 'non-EGS'($p<0.01$)
3. Statistically significant difference between horses with SGS and 'non-EGS' ($p<0.05$)

Horses with SGS had the highest mean level of IgA to BoNT/C in their ileum contents, followed by horses with CGS and then horses with AGS; horses without grass sickness had the lowest mean IgA level to BoNT/C (Table 6.2). There was a statistically significant higher level of IgA to BoNT/C in the ileum contents of the

horses with SGS compared to the horses without grass sickness ($p<0.05$). When the results for horses with CGS and SGS were compiled as a group, there was a significantly higher level of IgA to BoNT/C in the ileum contents of these horses compared to horses without grass sickness ($p<0.05$) (Fig. 6.7). When all horses with grass sickness were considered as a group, although the mean IgA level to BoNT/C was higher in these horses compared to horses without grass sickness, this difference was not statistically significant.

Table 6.2: IgA to *C. novyi* type A surface antigens and BoNT/C in ileum contents of horses with CGS, SGS, AGS and horses without grass sickness (non-EGS)

Statistics		Antibody levels OD_{405nm}			
		CGS (n=5)	**SGS (n=6)**	**AGS (n=9)**	**Non-EGS (12)**
IgA to BoNT/C	**Mean**	0.2	0.25[1]	0.15	0.12
	SD	0.14	0.12	0.09	0.05
	Range	0.04-0.35	0.05-0.37	0.07-0.31	0.07-0.22
IgA to surface antigens	**Mean**	0.09	0.13	0.08	0.09
	SD	0.04	0.07	0.08	0.04
	Range	0.04-0.14	0.06-0.24	0.02-0.28	0.05-0.18

[1]Statistically significant difference between horses with SGS and 'non-EGS' ($p<0.05$)

Horses with SGS had a higher mean level of IgA to the surface antigens in their ileum contents compared to horses with CGS, AGS or horses without grass sickness (Table 6.2). However, there were no statistically significant differences in the IgA levels to the surface antigens in the ileum contents between the different groups of horses.

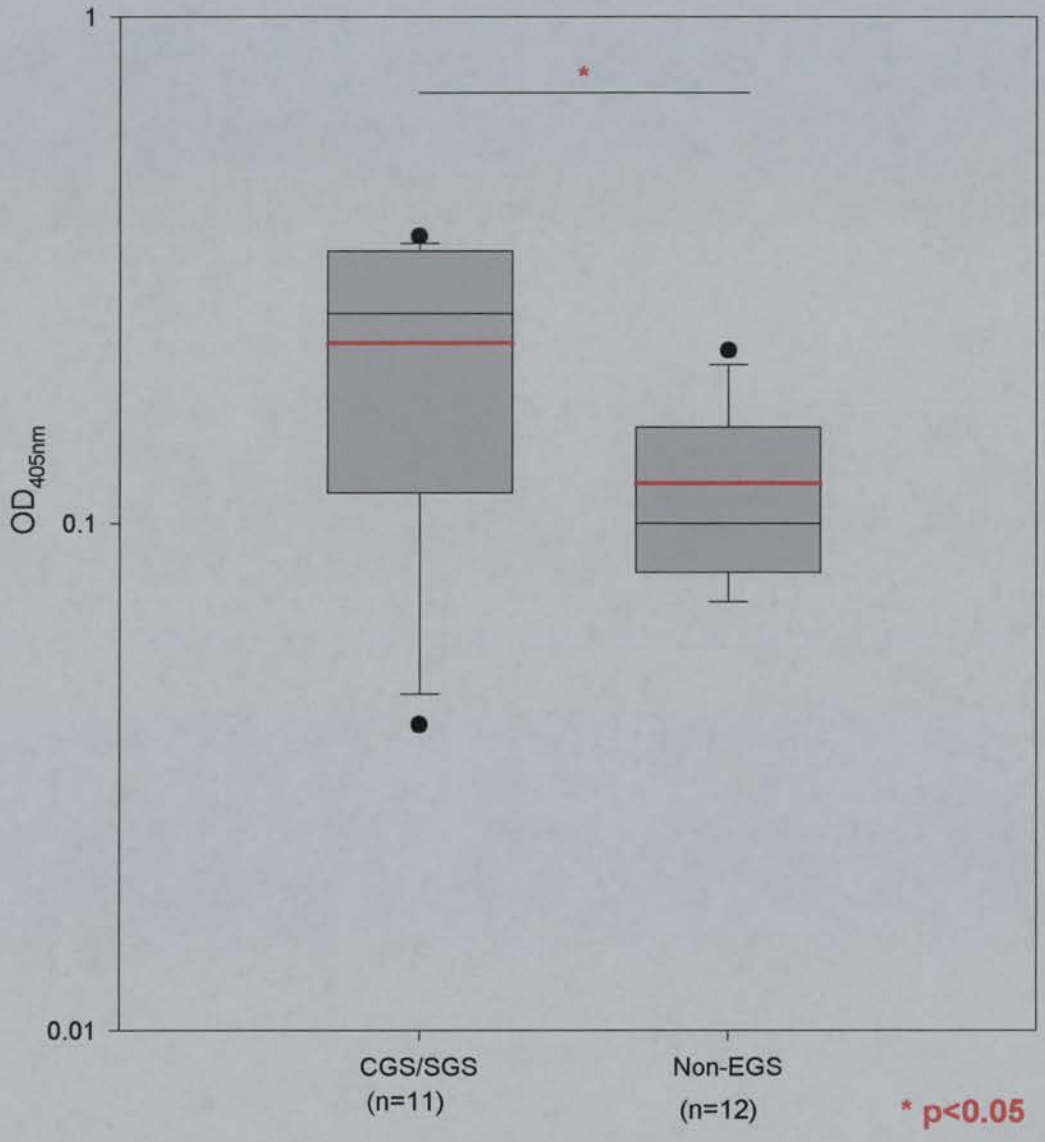


Figure 6.7: IgA to BoNT/C in ileum contents of horses with CGS or SGS and horses without EGS.

There was a higher mean level of IgA to BoNT/C in the faeces, compared to the ileum contents in horses with SGS and AGS; horses with CGS had a lower level of IgA to BoNT/C in the faeces; horses without grass sickness had the same mean level in the faeces and ileum contents (Tables 6.1 and 6.2). There was a higher mean level of IgA to *C. novyi* type A surface antigens in the faeces of all the horses compared to the ileum contents (Tables 6.1 and 6.2). There was also a higher mean level of IgA to BoNT/C, compared to the surface antigens, in the faeces and ileum contents of all the horses (Tables 6.1 and 6.2).

The 48 faecal control samples were collected from 33 healthy controls, seven horses with GI-associated disease, and eight horses with non-GI associated disease. The horses with GI-associated disease had the highest mean level of IgA to BoNT/C in the faeces (Table 6.3a). However, there were no statistically significant differences in IgA levels either to BoNT/C or to the surface antigens in the faeces between the three groups of control horses. Ileum samples were collected from two healthy controls, three horses with GI-associated disease and seven horses with non-GI associated disease. The healthy controls had the highest mean IgA level both to BoNT/C and to the surface antigens (Table 6.3b). However, there were no statistically significant differences in specific IgA to these antigens in the ileum contents between the three groups of control horses.

Table 6.3: IgA to BoNT/C and to *C. novyi* type A surface antigens in (a) faeces, and (b) ileum contents of control horses.

(a)

Statistics		Antibody levels OD_{405nm}		
		Healthy controls (n=33)	GI controls (n=7)	Non-GI controls (n=8)
IgA to BoNT/C	**Mean**	0.11	0.19	0.13
	SD	0.04	0.10	0.07
	Range	0.04-0.18	0.08-0.33	0.05-0.27
IgA to surface antigens	**Mean**	0.09	0.11	0.11
	SD	0.06	0.05	0.08
	Range	0.02-0.29	0.04-0.18	0.03-0.27

(b)

Statistics		Antibody levels OD_{405nm}		
		Healthy controls (n=2)	GI controls (n=3)	Non-GI controls (n=7)
IgA to BoNT/C	**Mean**	0.18	0.11	0.11
	SD	0.06	0.06	0.05
	Range	0.14-0.22	0.07-0.17	0.07-0.20
IgA to surface antigens	**Mean**	0.14	0.08	0.09
	SD	0.06	0.05	0.04
	Range	0.09-0.18	0.05-0.14	0.05-0.16

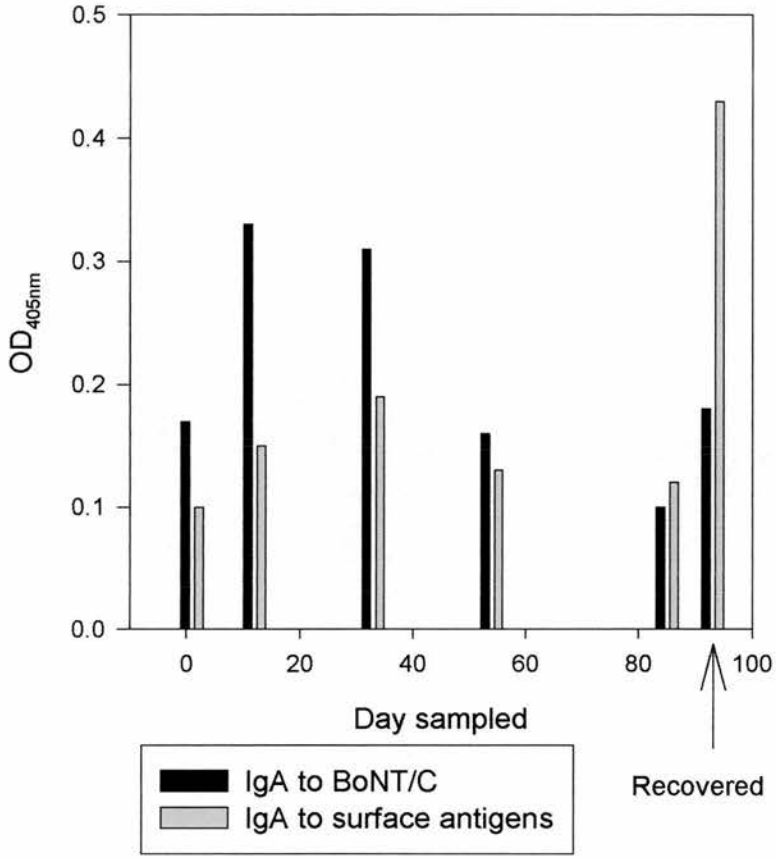
IgA to *C. novyi* type A surface antigens and BoNT/C in consecutive faecal samples from horses with CGS

Faecal samples were collected, on more than one occasion, from ten horses with CGS during the course of the disease: eight horses were sampled twice, one horse was sampled six times and one horse three times (Fig. 6.8). Six of the ten horses showed an increase in OD both to the surface antigens and to BoNT/C between the first and second faecal sample (Fig. 6.8a, c, d, e, g, and j); two of the ten horses showed an increase in OD to the surface antigens only (Fig. 6.8b and f). The horse that was sampled six times during the 93-day duration of disease showed a fluctuation in specific IgA levels to the antigens. IgA to BoNT/C peaked in the second sample collected at day 12, then began to decrease at days 33, 54 and 83, before increasing at day 93 (Fig. 6.8a). IgA to the surface antigens in this horse followed a similar pattern, but levels peaked at day 93 (Fig. 6.8a); the horse recovered. Three of the horses that showed an increase in IgA both to the surface antigens and BoNT/C recovered and three were euthanased. The two horses that showed an increase in IgA only to the surface antigens recovered. Of the two horses that showed a decrease in IgA both to surface antigens and to BoNT/C, one recovered and one was euthanased (Fig. 6.8h and i).

Comparison of specific IgA levels between horses with CGS that recovered and those that were euthanased.

There was a higher mean level of IgA to the surface antigens in horses that had recovered from CGS compared to those that were euthanased. However, there was a lower mean level of IgA to BoNT/C in the horses that recovered from CGS compared to those that were euthanased (Table 6.4). These differences are not statistically significant. When there was more than one sample for a particular

(a)



(b)

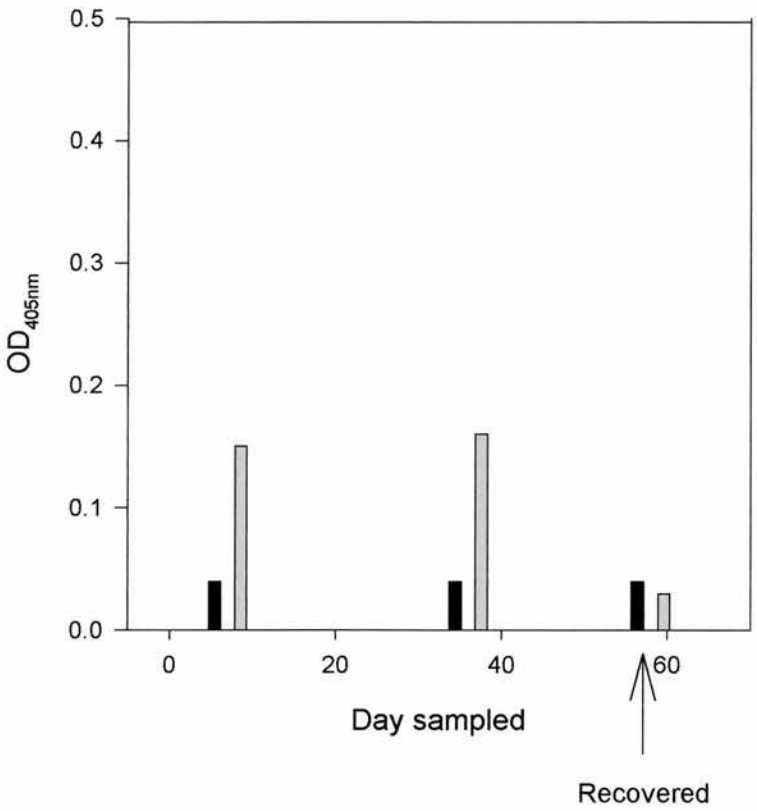
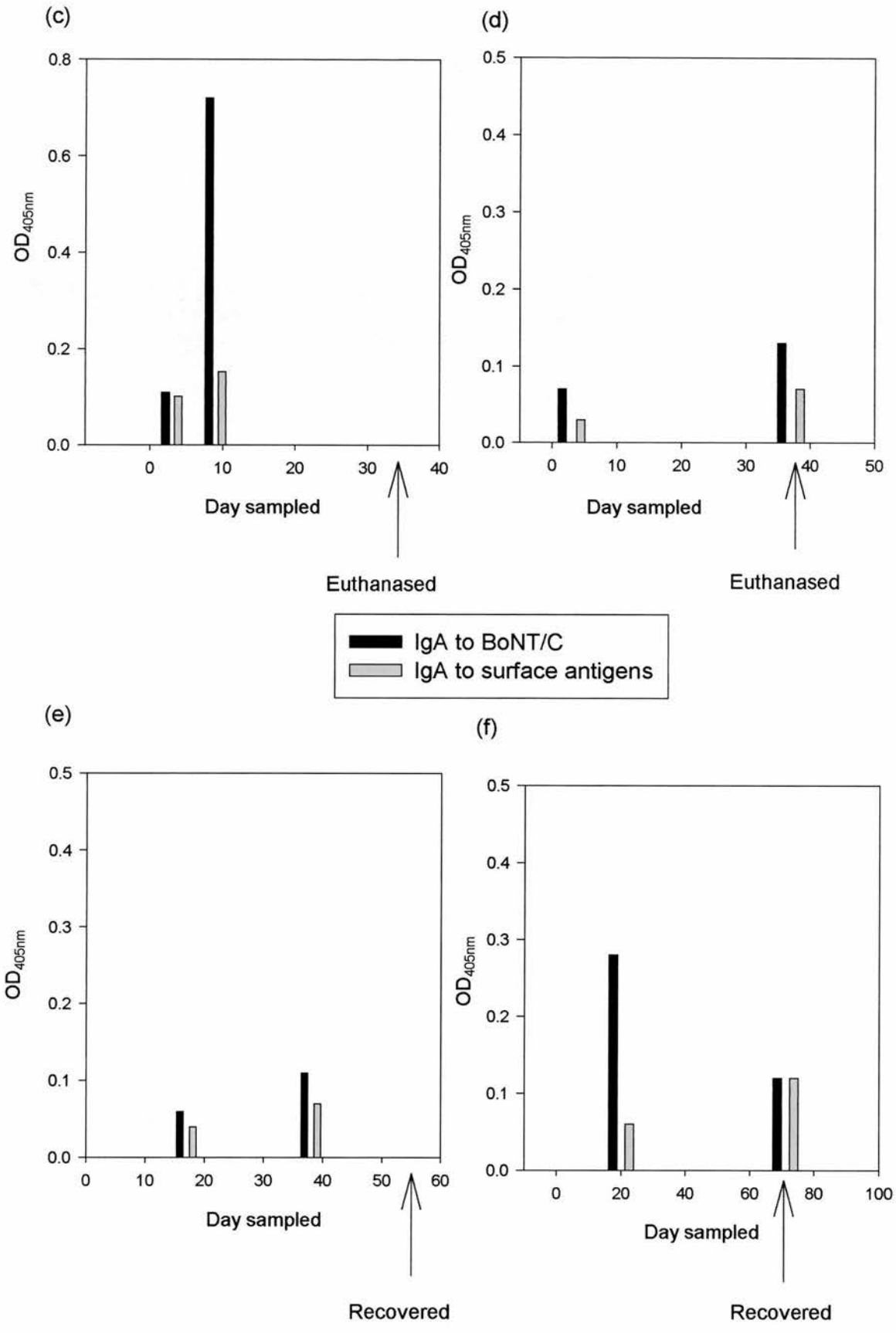
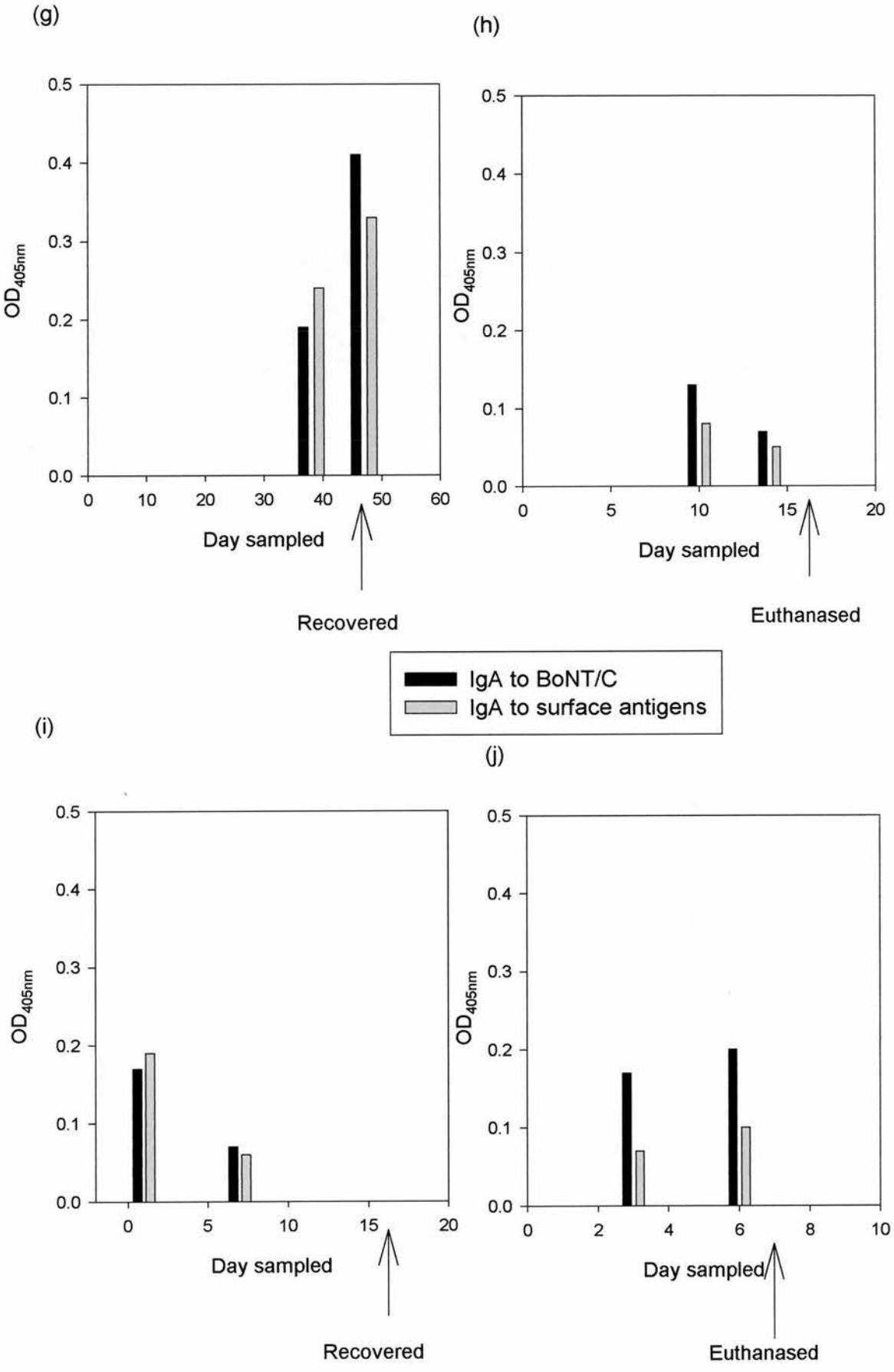


Figure 6.8(a)-(j): IgA to *C. novyi* type A and BoNT/C in the faeces of ten horses with CGS, sampled more than once over the course of the disease.


(c)
(d)
0.8
0.6
0.4
0.2
0.0
0.5
0.4
0.3
0.2
0.1
0.0
OD405nm
0 10 20 30 40
0 10 20 30 40 50
Day sampled
Euthanased
IgA to BoNT/C
IgA to surface antigens
(e)
(f)
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40 50 60
0 20 40 60 80 100
Recovered


(g)
(h)
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OD405nm
0 10 20 30 40 50 60
Day sampled
Recovered
0 5 10 15 20
Day sampled
Euthanased
IgA to BoNT/C
IgA to surface antigens
(i)
(j)
0 5 10 15 20
Day sampled
Recovered
0 2 4 6 8 10
Day sampled
Euthanased

horse, the mean OD of the samples was calculated. Fig. 6.8 shows the variation in OD values between these samples.

Table 6.4: IgA to *C. novyi* type A surface antigens and BoNT/C in the faeces of horses with CGS that recovered and those that were euthanased.

Horses with CGS	Statistics	Antibody levels OD_{405nm}	
		IgA to surface antigens	IgA to BoNT/C
Recovered (n=7)	**Mean**	0.13	0.15
	SD	0.08	0.08
	Range	0.05-0.29	0.04-0.3
Euthanased (n=8)	**Mean**	0.10	0.17
	SD	0.05	0.12
	Range	0.04-0.18	0.04-0.42

The IgA levels to the surface antigens and to BoNT/C in faeces, collected at the start of the disease and the end of the disease, were compared between horses with CGS that recovered and those that were euthanased. There was a higher level of IgA to the surface antigens and to BoNT/C both at the start (Table 6.5a) and end of the disease (Table 6.5b) in horses that eventually recovered, compared to those that were euthanased. However, these comparisons are based on a small number of samples, and the differences are not statistically significant. The horses used to calculate the levels of IgA at the start of the disease are not the same horses used for calculation of IgA levels at the end of the disease.

Table 6.5: IgA to *C. novyi* type A surface antigens and to BoNT/C in (a) faeces collected at the start of the disease, and (b) faeces collected at the end of the disease, from horses that recovered from CGS and those that were euthanased.

(a)

Horses with CGS	Statistics	Antibody levels OD_{405nm}	
		IgA to surface antigens	IgA to BoNT/C
Recovered (n=2)	**Mean**	0.15	0.17
	SD	0.06	0
	Range	0.1-0.19	0.17-0.17
Euthanased (n=4)	**Mean**	0.07	0.11
	SD	0.03	0.05
	Range	0.03-0.1	0.07-0.17

(b)

Horses with CGS	Statistics	Antibody levels OD_{405nm}	
		IgA to surface antigens	IgA to BoNT/C
Recovered (n=4)	**Mean**	0.23	0.19
	SD	0.19	0.16
	Range	0.03-0.45	0.04-0.41
Euthanased (n=4)	**Mean**	0.10	0.16
	SD	0.06	0.09
	Range	0.04-0.18	0.04-0.25

IgA to surface antigens and BoNT/C in the faeces of healthy horses sampled over 12 weeks.

Faecal samples from six healthy control horses were collected three times over a 12-week period, at weeks one, four and 12. Fluctuations were observed in the IgA levels detected both to BoNT/C and the surface antigens between the three faecal samples collected from each horse (Fig. 6.9). The pattern of fluctuation of IgA to BoNT/C did not follow the same pattern for IgA to the surface antigens. The changes in IgA to BoNT/C were not the same for all six horses. Two of the horses showed a decrease in IgA to BoNT/C in the second faecal sample, followed by an increase in the third sample (Fig. 6.9a and b). Two horses showed an increase in IgA to BoNT/C in both the second and third faecal samples compared to the first (Fig. 6.9c and d). One horse showed little variation in IgA to BoNT/C between the first and second samples, but an increase in the third faecal sample (Fig. 6.9e). The sixth horse showed an increase in IgA to BoNT/C between the first and second sample, with little change in the third (Fig. 6.9f). All six of the horses showed an increase in IgA to the surface antigens in the second sample followed by a decrease in the third faecal sample (Fig. 6.9a-f). However, all these observed changes reflect quite small changes in OD measurements.

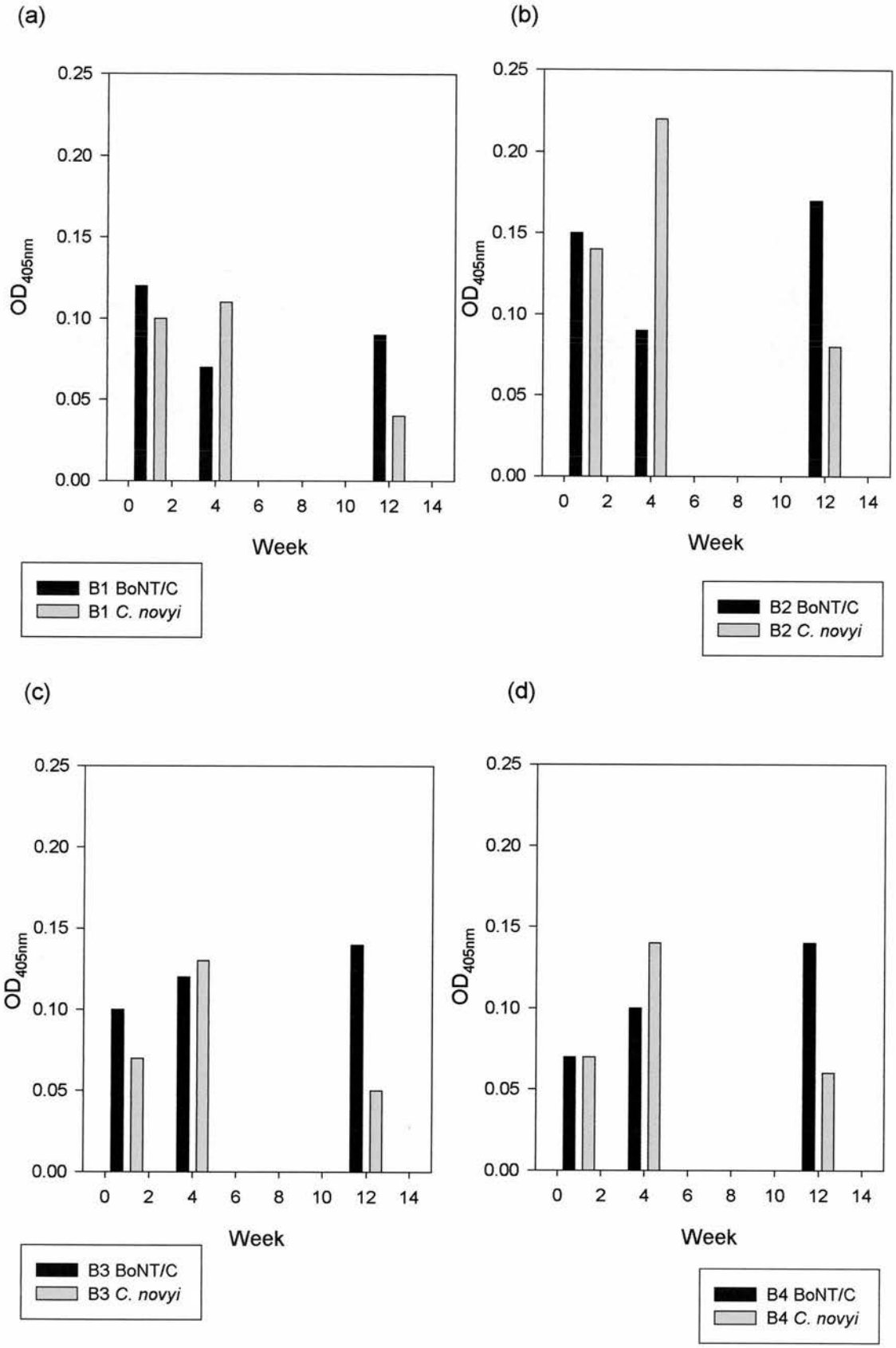
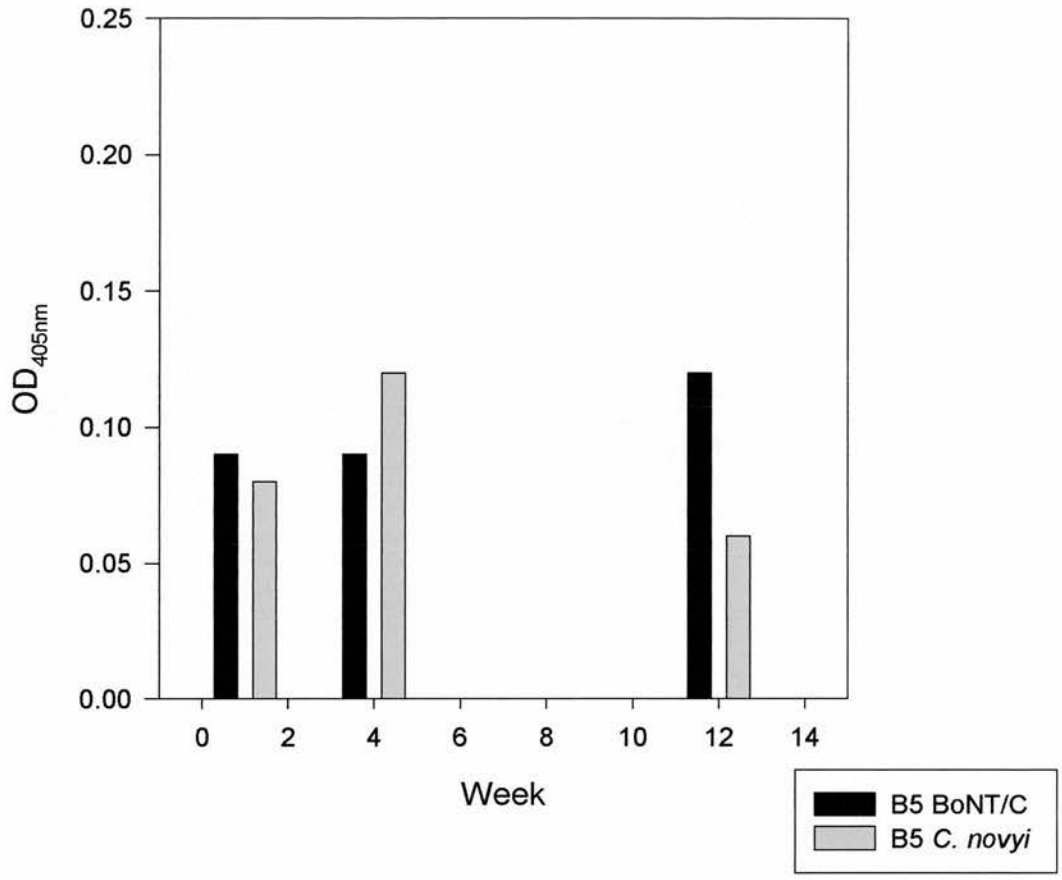


Figure 6.9 (a)-(f): IgA to *C. novyi* type A surface antigens and BoNT/C in the faeces of six healthy control horses sampled over a period of 12 weeks.

(e)


0.25
0.20
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6
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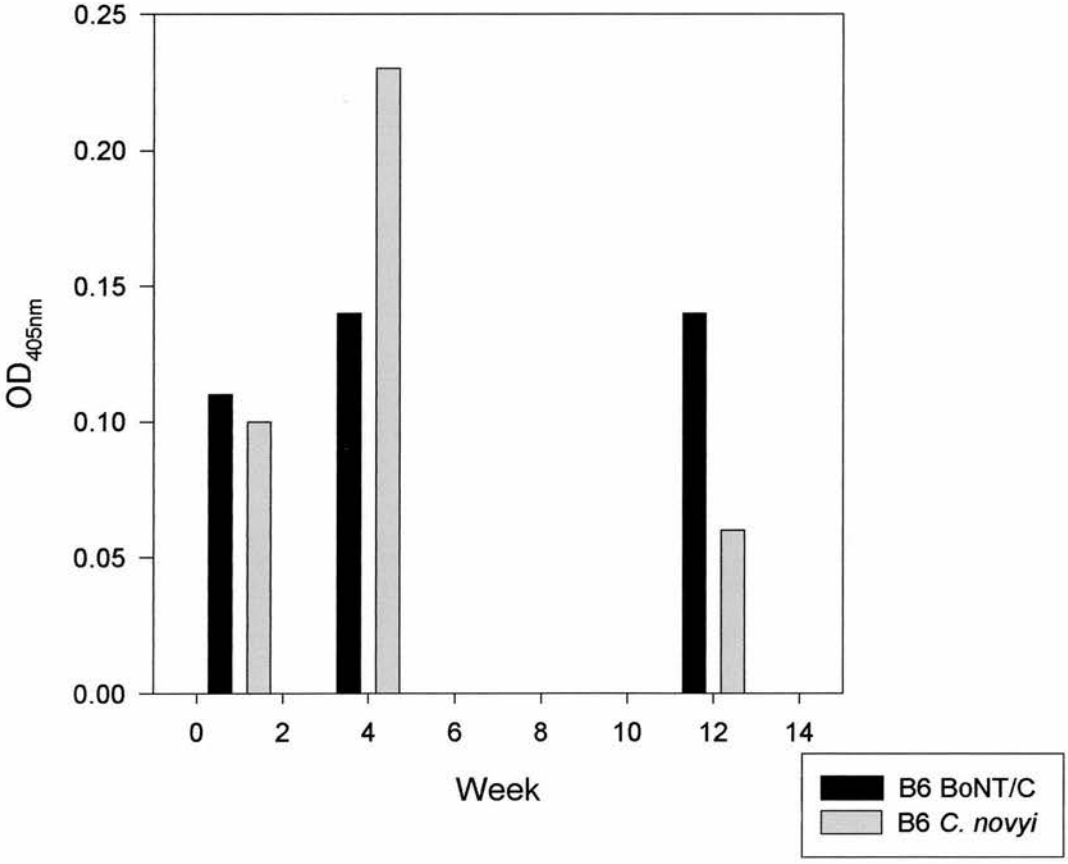
14
Week
B5 BoNT/C
B5 C. novyi

(f)


0.25
0.20
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OD405nm
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6
8
10
12
14
Week
B6 BoNT/C
B6 C. novyi

6.1.3 Flow cytometry results

Flow cytometry results for equine PBMCs

Lymphocyte and monocyte populations for equine PBMCs were identified based on the bitmap formed by forward and side scatter of light, reflecting the size and granularity of the cells (Fig. 6.10) (Sharpe, 1988). Initial assays were carried out to determine the optimum dilution and volume of primary antibodies to use for the detection of T and B cell populations in equine PBMCs: anti-CD3 for the detection of equine T cells and CVS 36 for the detection of equine B cells. PBMCs were isolated from two different horses and labelled with 40μl of anti-equine CD3 and CVS 36; the primary antibodies were used undiluted and at dilutions of 1 in 10 and 1 in 20 dilutions. PBMCs were also labelled with anti-CD18, an antibody that binds to all leucocytes.

In experiment one, 21% of the PBMCs from both horses were in the lymphocyte population and 21-22% were in the monocyte population (Table 6.6). There was little difference in the percentage of cells in the lymphocyte population positively labelled with the different dilutions of anti-CD3 (Table 6.7). However, there was a significant drop in percentage of cells positively labelled when CVS 36 was used at either 1 in 10 or 1 in 20 dilutions, compared to the undiluted antibody (Table 6.7), suggesting that it should be used undiluted for future assays.

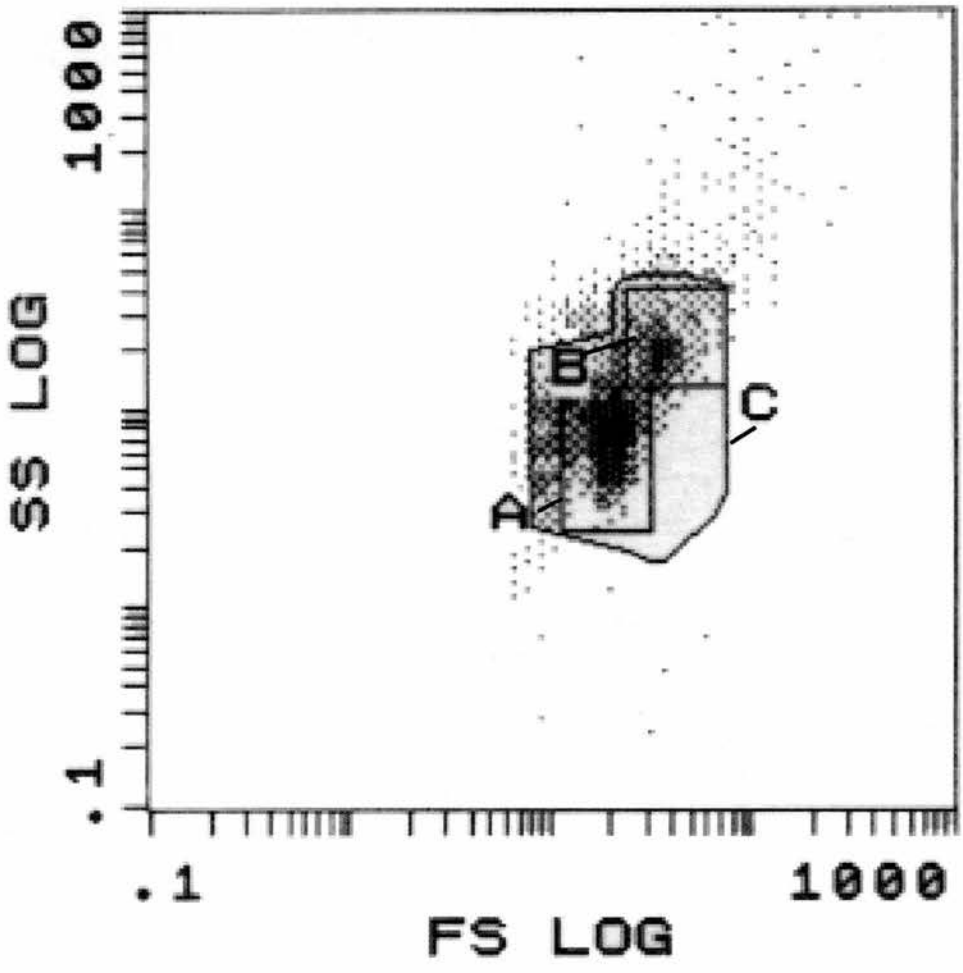


Figure 6.10: Flow cytometric analysis of PBMCs. Cell populations identified by forward and side scatter of light. Population A respresents the lymphocyte population and population B the monocyte population.

Table 6.6: Flow cytometric analysis of PBMCs from a horse with chronic grass sickness (horse 1) and a healthy control (horse 2), showing the percentage of lymphocytes and monocytes in PBMCs.

Cells	Percentage of cells	
	In lymphocyte region	In monocyte region
PBMCs of horse 1	21.4	22.7
PBMCs of horse 2	21.6	21.1

Table 6.7: Flow cytometric analysis of PBMCs from a horse with chronic grass sickness (horse 1) and a healthy control (horse 2), showing the percentage of cells in the lymphocyte population positively labelled with anti-CD18, and different dilutions of anti-CD3 and CVS 36 (experiment one).

Primary antibody dilutions		Percentage positively labelled lymphocytes	
		Horse 1	Horse 2
Anti-CD3	**Undiluted**	83.8	66.6
(T cells)	**1 in 10**	73.9	70.8
	1 in 20	81.0	69.9
CVS 36	**Undiluted**	21.4	37.4
(B cells)	**1 in 10**	5.8	8.6
	1 in 20	4.0	8.1
Anti-CD18	**1 in 10**	76.8	81.6
(all leucocytes)			

When anti-equine CD3 was used undiluted, 84% of cells in the lymphocyte population of the horse with CGS (horse one) and 67% of cells in the lymphocyte population of the healthy control (horse two) were positively labelled (Table 6.7). CVS 36 labelled 22% of cells in the lymphocyte population of horse one compared to 37% of cells in the lymphocyte population of horse two (Table 6.7). The anti-CD18 antibody detected 77% of cells in the lymphocyte population of horse one and 82% of cells in the lymphocyte population of horse two (Table 6.7).

The anti-equine CD3 and CVS 36 antibodies also positively labelled a high percentage of the cells in the monocyte population (Table 6.8). CVS 36 positively labelled a higher percentage of cells in the monocyte population than in the lymphocyte population in both horses. The anti-CD18 antibody detected 98-99% of cells in the monocyte population of the two horses (Table 6.8).

Table 6.8: Percentage of positively labelled cells with anti-CD18, and different dilutions of anti-CD3 and CVS 36, in the monocyte population of PBMCs from a horse with CGS (horse 1) and a healthy control (horse 2) (experiment one).

Primary antibody dilutions		% of positively labelled monocytes	
		Horse 1	Horse 2
Anti-CD3	**Undiluted**	82.1	61.4
(T cells)	**1 in 10**	80.0	63.6
	1 in 20	89.9	66.8
CVS 36	**Undiluted**	75.9	79.5
(B cells)	**1 in 10**	48.0	36.8
	1 in 20	26.5	32.3
Anti-CD18	**1 in 10**	98.7	97.9
(all leucocytes)			

The high percentage of positively labelled cells with anti-B and T cell antibodies in the monocyte populations of both horses in experiment one may be due to non-specific binding of primary antibodies by monocytes. A blocking step was included in experiment two to compare labelling of blocked and unblocked cells with anti-CD3 and CVS 36 in the lymphocyte and monocyte populations.

The horse in experiment two had a higher percentage of lymphocytes and a lower percentage of monocytes in the PBMCs (Table 6.9) compared to both horses in experiment one (Table 6.6). Blocking of cells, with normal goat serum for 15 min at 4°C, reduced non-specific binding to some extent. Both anti-CD3 and CVS36 antibodies stained a slightly higher percentage of cells in the lymphocyte gate, and a

slightly lower percentage of cells in the monocyte gate, when the cells were blocked (Table 6.10). The blocking step was included in further experiments.

Table 6.9: Flow cytometric analysis of PBMCs from a control horse showing the percentage of lymphocytes and monocytes in PBMCs when cells were blocked and not blocked (experiment two).

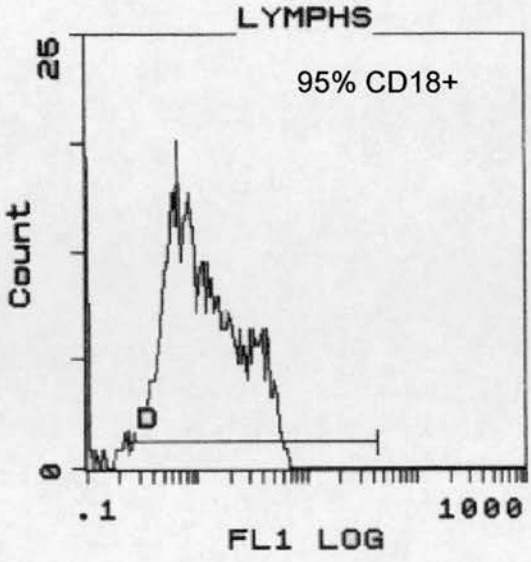
Cells	Percentage of cells	
	In lymphocyte region	In monocyte region
PBMCs with blocking	61.5	15.9
PBMCs without blocking	68.7	9.9

Table 6.10: Effects of blocking on the percentage of cells in the lymphocyte and monocyte populations of PBMCs isolated from a control horse positively labelled with anti-CD3, CVS 36 and anti-CD18 (experiment two).

Primary antibody dilutions		Percentage of positively labelled cells			
		Blocked		Not blocked	
		Lymphocyte population	Monocyte population	Lymphocyte population	Monocyte population
Anti-CD3	**Undiluted (10μl)**	83.4	39.6	81.8	41.8
	1 in 10 dilution (10μl)	60.7	9.4	68.7	16.5
CVS 36	**Undiluted (40μl)**	13.8	23.2	12	30
	Undiluted (10μl)	12	17.8	9.7	23.2
Anti-CD 18		95	90.5	92.6	94.0

Use of a lower volume of undiluted anti-CD3 (10μl as opposed to 40μl) did not reduce the percentage of positively labelled cells detected in the lymphocyte population (Table 6.7 and 6.10). However, the percentage of positively labelled cells in the monocyte population was reduced when a lower volume of anti-CD3 was used (Tables 6.8 and 6.10). The 1 in 10 dilution of anti-CD3 reduced both the percentage of positively labelled cells in the lymphocyte and monocyte regions, compared to the undiluted antibody (Table 6.10). On this basis, 10μl of undiluted anti-CD3 antibody was used in further experiments. Use of a lower volume of CVS 36, 10μl as opposed to 40μl, also lowered the percentage of positively labelled cells in both the lymphocyte and monocyte regions (Table 6.10); 40μl of undiluted CVS 36 antibody was used in further experiments. A higher percentage of cells in the monocyte population were positively labelled with the CVS 36, compared to cells in the lymphocyte population, regardless of whether the cells were blocked or not. When the cells were blocked, 95% of the cells in the lymphocyte population were positively labelled with anti-CD18, 83% positively labelled with anti-CD3 and 14% positively labelled with CVS 36 (Fig. 6.11a, c and e). When the cells in the lymphocyte population were labelled with anti-CD18, two peaks of fluorescence were observed (Fig. 6.11a), indicating the presence of two populations of cells. One fluorescence peak was observed with monocytes labelled with anti-CD18; this peak was shifted to the right in comparison to the labelled lymphocytes, indicating a greater intensity of fluorescence with CD18+ monocytes (Fig. 6.11b). A diffuse population of lymphocytes were labelled with CVS 36 as illustrated by the intensity of fluorescence in these labelled cells (Fig. 6.11c). The CVS 36 monocytes appeared to have a more defined population of cells positively labelled, but the actual numbers of positively labelled cells were much lower in this population (Fig. 6.11d). The anti-CD3 antibody labelled a discrete population of T cells in the

(a)



(b)

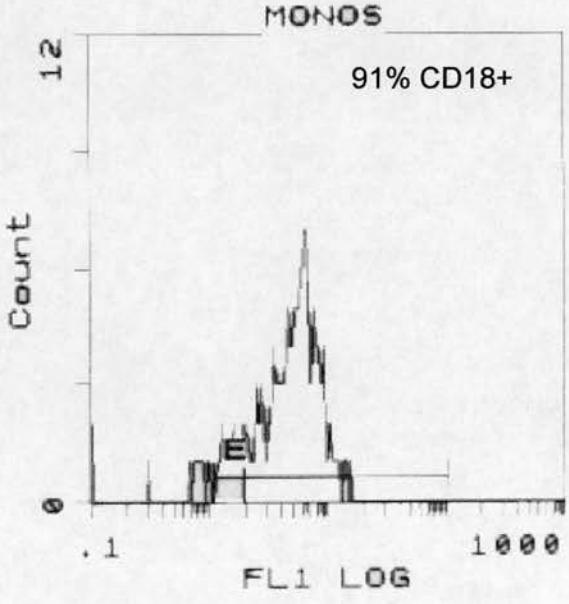
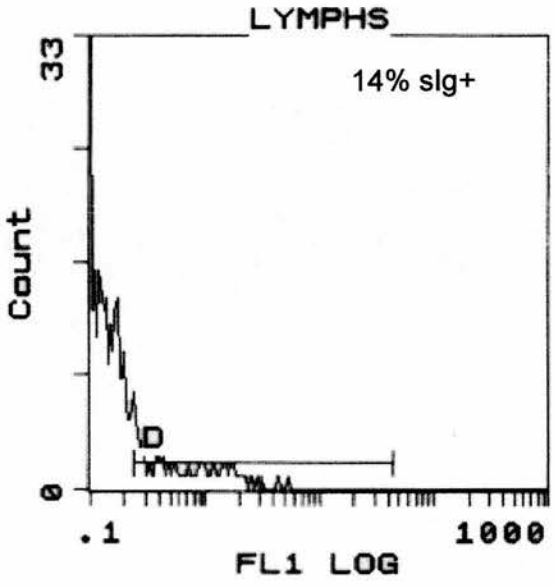


Figure 6.11: Flow cytometric analysis of equine PBMCs labelled with anti-CD18, anti-CD3 and CVS 36. Plots of cell number per channel versus relative fluoresence intensity of cells labelled with antibodies.
(a) Cells in the lymphocyte population and (b) cells in the monocyte population positively labelled with anti-CD18. (c) Cells in the lymphocyte population and (d) cells in the monocyte population positively labelled with CVS 36 (sIg+). (e) Cells in the lymphocyte population and (f) cells in the monocyte population positively labelled with anti-CD3.
Gates were set to approximately 1% fluoresence with negative controls. The mean percentage of positive cells is indicated in each plot.

(c)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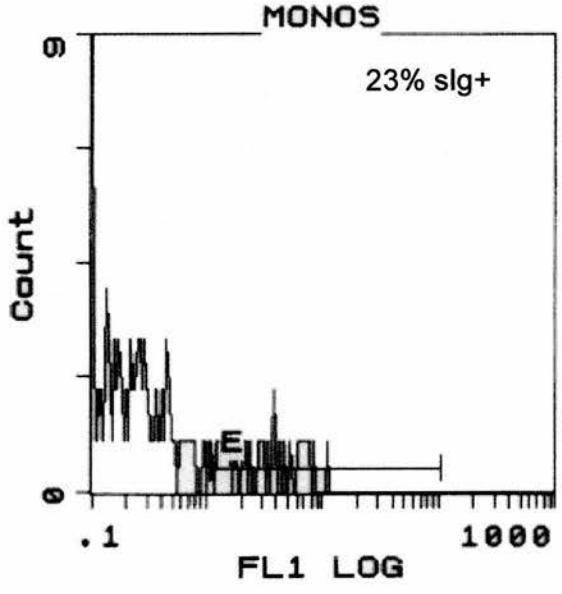
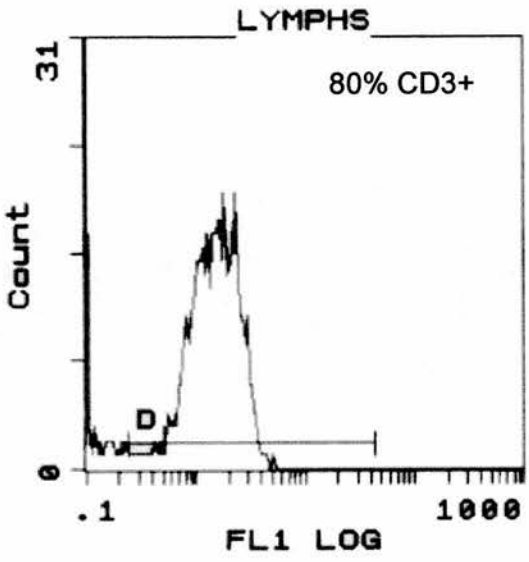
14% slg+
33
Count
0
D
.1
1000
FL1 LOG

(d)


MONOS
23% slg+
9
Count
0
E
.1
1000
FL1 LOG

(e)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31
Count
0
D
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1000
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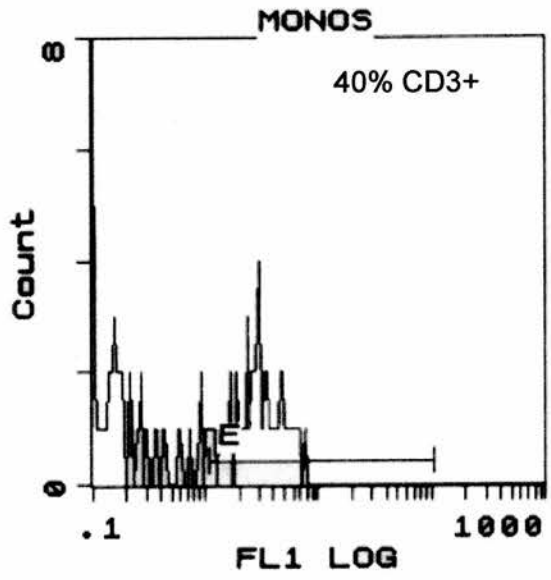
(f)


MONOS
40% CD3+
8
Count
0
E
.1
1000
FL1 LOG

lymphocyte population (Fig. 6.11e); the anti-CD3 antibody labelled a population of monocytes with similar fluorescence intensity but the actual numbers of cells labelled were much lower than in the lymphocyte population (Fig. 6.11f).

Flow cytometry results for cells isolated from equine GALT

Lymphocyte and monocyte populations isolated from the lamina propria and Peyer's patches of the equine gastric associated lymphoid tissue (GALT), were identified based on the bitmap formed by forward and side scatter of light (Fig. 6.12). Cells were collected both during and after the removal of the epithelium and after collagenase digestion of either the lamina propria or Peyer's patches in order to compare the cell populations recovered at different stages in the isolation procedure. For these experiments, 10μl of undiluted anti-CD3, 40μl of undiluted CVS 36 and 10μl of anti-CD18 (diluted 1 in 10) were used as determined by the experiments with equine PBMCs.

In experiment three, cells were isolated from the lamina propria of a horse with SGS, and collected after the 6th and 8th (final) EDTA wash to remove the epithelium, and after collagenase digestion of the lamina propria. The percentage of cells in the lymphocyte region increased from 15% after the 6th EDTA wash, to 19% after the 8th (final) EDTA wash, reaching 43% after collagenase digestion (Table 6.11). The percentage of monocytes decreased from 22% in the final wash with EDTA to 7% after collagenase digestion (Table 6.11). The percentage of cells in the lymphocyte population that were positively labelled with anti-equine CD3 increased from 22% after the 6th EDTA wash, to 49% after the final EDTA wash, and 62% after the collagenase digestion (Table 6.12). There was little difference in the percentage of cells positively labelled with CVS 36 between the 6th and 8th EDTA wash; only 3% of

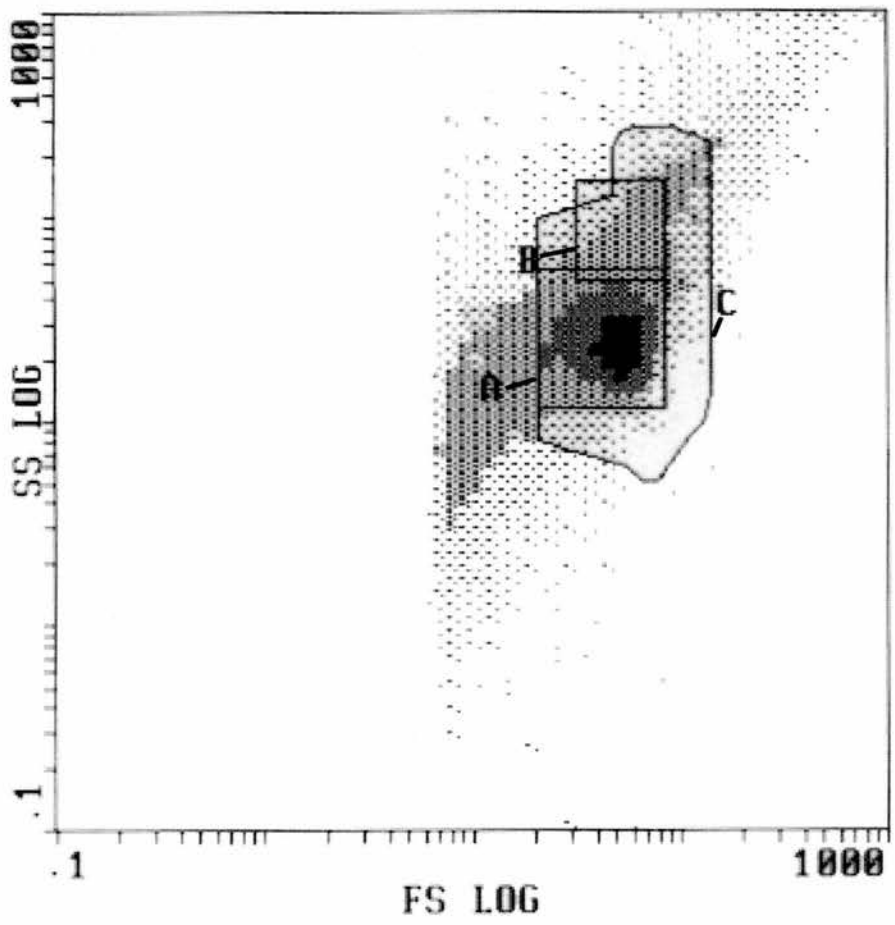


Figure 6.12: Flow cytometric analysis of cells isolated from the lamina propria. Cell populations identified by forward and side scatter of light. Population A represents the lymphocyte population and population B the monocytes.

cells were positive after the 6th wash and 2% positive after the final EDTA wash (Table 6.12). The increase in positively labelled cells after collagenase digestion was very small, with only 5% of cells positive for CVS 36 (Table 6.12). In experiment three, there was a higher percentage of cells positively labelled with the CVS 36 antibody in the monocyte population compared to cells in the lymphocyte population (Table 6.12).

Table 6.11: Flow cytometric analysis of cells isolated from the lamina propria of the GALT from a horse with SGS. Percentage of lymphocytes and monocytes in cells collected during and after removal of epithelium with EDTA, and after collagenase digestion of lamina propria (experiment three).

Isolation stage	**Percentage of cells**	
	In lymphocyte region	**In monocyte region**
After 6th EDTA wash	14.9	11.7
After 8th EDTA wash (final wash)	18.6	22.2
After collagenase digestion	42.7	7.2

Table 6.12: Flow cytometric analysis of cells isolated from the lamina propria of the GALT from a horse with SGS. The percentage of cells in the lymphocyte and monocyte populations that were positively labelled with anti-CD3, CVS 36 and anti-CD18 antibodies (experiment three).

Isolation stage	Primary antibody	Percentage of positively labelled cells	
		Lymphocytes	Monocytes
EDTA wash 6	**Anti-CD3**	21.6	4.9
	CVS 36	3.4	9.8
EDTA wash 8 (final EDTA wash)	**Anti-CD3**	49.3	3.5
	CVS 36	2.3	3.5
After collagenase digestion	**Anti-CD3**	61.8	31.8
	CVS 36	4.8	8.7
	Anti-CD18	55	28.6

In experiment four, cells were isolated from both the lamina propria and a Peyer's patch from a horse with glomerulonephritis (two separate sections of gut were collected). Monocytes were removed from the cells that had been isolated by collagenase digestion, through the ability of the monocytes to stick to a plastic surface. The cells were incubated in a plastic petri dish or tissue culture flask depending on volume, for one hour at 37°C with 5% CO_2. The cell suspension was mixed gently by swirling to resuspend any lymphocytes that had settled out and then the supernatant was poured off, leaving monocytes stuck to the plastic surface. Monocytes were removed using a cell scraper and resuspended in RPMI 1640, if required. The cells collected at different stages in the isolation procedure were again compared, and the effect of monocyte removal on the populations of cells investigated.

The cells isolated from the lamina propria after collagenase digestion had a higher percentage of cells in the lymphocyte population, and a lower percentage of cells in the monocyte population, compared to the cells recovered in the final wash with EDTA (Table 6.13). This was also the case for cells isolated after collagenase digestion of the Peyer's patch (Table 6.13). Removal of monocytes from cells isolated after collagenase digestion, followed by separation of the remaining cells on Histopaque, increased the percentage of cells in the lymphocyte population by 17% for cells from the lamina propria and 12% for cells from the Peyer's patches (Table 6.13). The percentages of cells in the monocyte populations were only decreased by 2% after monocyte removal and Histopaque separation of cells from both the lamina propria and Peyer's patch (Table 6.13). There was a higher percentage of cells in the lymphocyte region in the cells isolated after collagenase digestion of the Peyer's patch compared to those from the lamina propria: 65% of cells isolated from the Peyer's patch were lymphocytes, compared to 51% from the lamina propria (Table 6.13).

Table 6.13: Flow cytometric analysis of cells isolated from the lamina propria and Peyer's patch of the GALT of a horse with glomerulonephritis. Percentage of lymphocytes and monocytes in cells collected during and after removal of epithelium with EDTA, and after collagenase digestion of lamina propria (experiment four).

GALT	Stage of isolation procedure	Percentage of cells	
		In lymphocyte region	In monocyte region
LAMINA PROPRIA	10th EDTA wash	40.7	20.5
	13th EDTA wash (final wash)	38.4	16.5
	After collagenase digestion	51.4	8.5
	After collagenase digestion, removal of monocytes and isolation on histopaque	67.7	6.6
PEYER'S PATCHES	9th EDTA wash	32	24.4
	13th EDTA wash (final wash)	38.2	19.4
	After collagenase digestion	65.4	6.9
	After collagenase digestion, removal of monocytes and isolation on histopaque	76.9	4.5

There was a higher percentage of cells binding to anti-CD3 in the lymphocyte population of cells isolated after collagenase digestion of the lamina propria and Peyer's patch, compared to those recovered from the final EDTA wash (Table 6.14). However, there were also a higher percentage of cells binding to the anti-CD3 antibody in the monocyte population, in the cells isolated from the lamina propria and Peyer's patch (Table 6.14). CVS 36 antibody bound to a higher percentage of cells in both the lymphocyte and monocyte populations after collagenase digestion of the lamina propria compared to the final EDTA wash (Table 6.14). In cells

isolated from the Peyer's patch, there was a 2% increase in binding of CVS 36 to cells in the lymphocyte population, and a 4% decrease in the binding of the antibody to cells in the monocyte population after collagenase digestion compared to cells recovered from the final EDTA wash (Table 6.14).

Removal of monocytes and separation by Histopaque of the remaining cells from the collagenase digestion of the lamina propria resulted in an increase in the percentage of cells in the lymphocyte population positively labelled with anti-CD3 from 58 to 74% of cells (Table 6.14). There was also a 2% increase in the percentage of cells in the monocyte population labelled with anti-CD3 from 16 to 18% (Table 6.14). A lower percentage of cells in both the lymphocyte and monocyte populations were stained positive with CVS 36 after removal of monocytes and separation on Histopaque of cells (Table 6.14). Similar results were found after the removal of monocytes and separation on Histopaque of cells from the Peyer's patch: there was an increase in the percentage of cells staining with anti-CD3 in the lymphocyte and monocyte populations and a decrease in the percentage of cells staining with CVS 36 in both populations (Table 6.14).

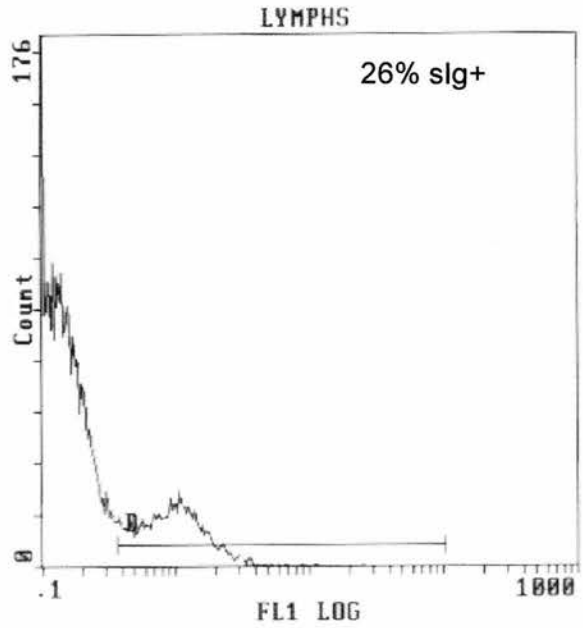
Table 6.14: Flow cytometric analysis of cells isolated from the lamina propria and Peyer's patch of the GALT of a horse with glomerulonephritis. The percentage of cells in the lymphocyte and monocyte populations positively labelled with anti-CD3 and CVS 36 antibodies (experiment four).

GALT	Stage in isolation procedure	Primary antibody	Percentage of positively labelled cells	
			In lymphocyte region	In monocyte region
LAMINA PROPRIA	**10th EDTA wash**	**Anti-CD3**	42.7	3.9
		CVS 36	19.4	47.5
	13th EDTA wash (final wash)	**Anti-CD3**	45.7	4.77
		CVS 36	19.8	48.5
	After collagenase digestion	**Anti-CD3**	58	16.2
		CVS 36	25.5	58.4
	After collagenase digestion, removal of monocytes and isolation on histopaque	**Anti-CD3**	73.7	18.3
		CVS 36	13.4	38.9
PEYER'S PATCHES	**9th EDTA wash**	**Anti-CD3**	47	3.3
		CVS 36	17.5	46.5
	13th EDTA wash (final wash)	**Anti-CD3**	49.3	3.1
		CVS 36	16.2	38.3
	After collagenase digestion	**Anti-CD3**	52.2	7.3
		CVS 36	18.1	34.1
	After collagenase digestion, removal of monocytes and isolation on histopaque	**Anti-CD3**	64.2	12.9
		CVS 36	12.6	29.2

There were higher percentages of cells positive for anti-CD3 and CVS 36 in the lymphocyte population of cells isolated from the lamina propria compared to cells from the Peyer's patch. In the cells isolated by collagenase digestion of the lamina propria, 58% of cells in the lymphocyte region stained positive with anti-CD3 and 26% of cells stained positive with CVS 36 (Fig. 6.13). The CVS 36 antibody labelled a discrete population of cells in the lymphocyte population (Fig. 6.13a). A more diffuse population of cells were labelled with the CVS 36 antibody in the monocyte population – one population had a similar fluorescence intensity to the CVS36+ lymphocytes, but there were also cells with a greater intensity of fluorescence in the monocyte population (Fig. 6.13b). However, fewer cells were fluorescent in the monocyte population. The anti-CD3 antibody labelled a discreet population of cells in the lymphocyte population (Fig. 6.13c); this antibody labelled a less discreet population of cells in the monocyte population and fewer cells were fluorescent (Fig. 6.13d). In cells isolated from the Peyer's patch, 52% of cells in the lymphocyte population were positive for anti-CD3 and 18% were positive with CVS 36. There were also higher percentages of cells in the monocyte population of cells isolated from the lamina propria that stained positively with anti-CD3 and CVS 36, compared to cells from the Peyer's patch (Table 6.14).

In experiment five, cells were isolated from the lamina propria of the GALT from a horse that had been diagnosed with having equine motor neurone disease (EMND). The monocytes were again removed after collagenase digestion of the lamina propria, and the remaining cells were separated on Histopaque. In this experiment cells were compared before and after separation of the cells on Histopaque.

(a)



(b)

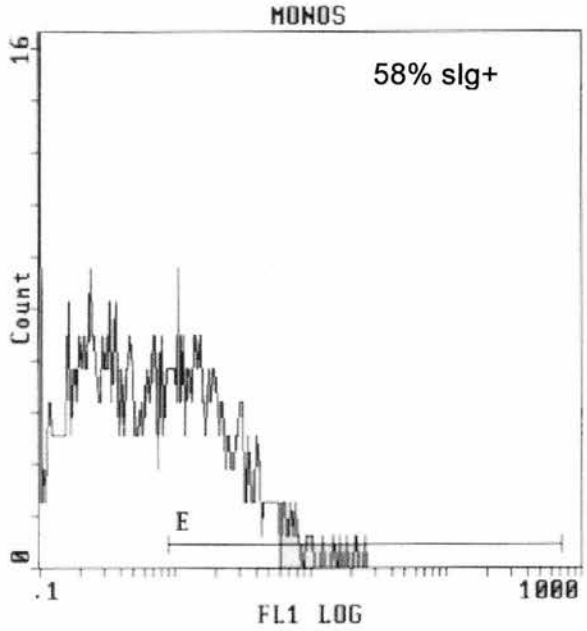
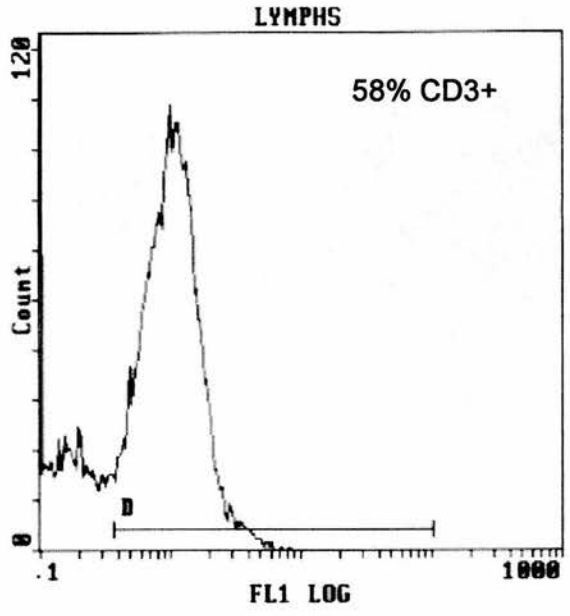
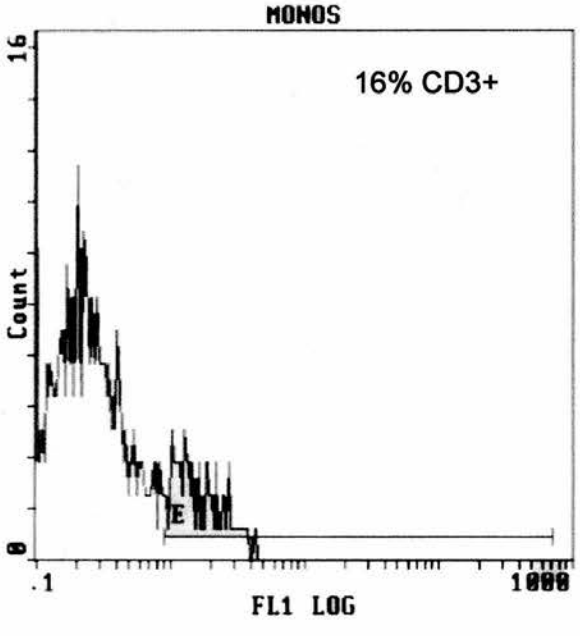


Figure 6.13: Flow cytometric analysis of cells isolated from the lamina propria of equine GALT positively labelled with anti-CD3 and CVS 36. Plots of cell number per channel versus relative fluoresence intensity of cells labelled with antibodies.
(a) Cells in lymphocyte population and (b) cells in monocyte population positively labelled with CVS 36 (sIg+). (c) Cells in lymphocyte population and (d) cells in monocyte population positively labelled with anti-CD3.
Gates were set to approximately 1% fluoresence with negative controls. The mean percentage of positive cells is indicated in each plot.

(c)


LYMPHS
58% CD3+
120
Count
0
D
.1
1000
FL1 LOG

(d)


MONOS
16% CD3+
16
Count
0
E
.1
1000
FL1 LOG

Removal of monocytes after collagenase digestion, resulted in 61% of the isolated cells falling in the lymphocyte region and 13% of cells falling in the monocyte region (Table 6.15). After separation on Histopaque, the percentage of cells in the lymphocyte region increased to 75% and the percentage of cells in the monocyte region decreased to 8% (Table 6.15). However, when the monocytes that had been removed were examined it was found that 60% of these cells fell into the lymphocyte region and only 7% in the monocyte region (Table 6.15).

Table 6.15: Flow cytometric analysis of the cells isolated from the lamina propria of the GALT of a horse with EMND, showing the percentage of cells in lymphocyte and monocyte regions (experiment five).

Cells	**Percentage of cells**	
	In lymphocyte region	**In monocyte region**
Lamina propria cells after removal of monocytes	61	13
Lamina propria cells after separation on Histopaque	75	8
Monocytes	60	7

Separation of the cells isolated by collagenase digestion, on Histopaque, increased the percentage of cells in the lymphocyte region positively labelled with anti-CD3 from 48% (after removal of the monocytes) to 70%; the percentage of cells positively staining with CVS 36 decreased from 24-14% (Table 6.16). The percentage of cells in the monocyte region binding either anti-CD3 or CVS 36 decreased after separation on Histopaque (Table 6.16). There were a similar percentage of cells,

staining positively with anti-CD3 and CVS 36, in the monocytes that were removed after collagenase digestion and in the cells from which they had been removed (Table 6.16).

Table 6.16: Flow cytometric analysis of the cells isolated from the lamina propria of the GALT of a horse with EMND. The percentage of cells in the lymphocyte and monocyte populations, positively labelled with anti-CD3 and CVS 36 antibodies, after the removal of monocytes and separation on Histopaque (experiment five).

Cells	Primary antibody	Percentage of positively labelled cells	
		Lymphocyte region	Monocyte region
Lamina propria cells after removal of monocytes	**Anti-CD3**	48	14
	CVS 36	24	68
Lamina propria cells after separation on Histopaque	**Anti-CD3**	70	10
	CVS 36	14	63
Monocytes	**Anti-CD3**	41	15
	CVS 36	22	62

In all three groups of cells, there were higher percentages of cells in the monocyte populations positively labelled with CVS 36 compared to the cells in the lymphocyte populations (Table 6.16). The differences in percentage of cells in the monocyte populations positively labelled with CVS 36 between the three groups reflected the differences in percentages of cells in the monocyte region between the three groups (Tables 6.15 and 6.16). For example, there was a 5% decrease in cells in the monocyte region after separation on Histopaque, and there was also a 5% decrease

in percentage of cells staining with CVS 36 in the monocyte region after separation on Histopaque.

6.1.4 ELISPOT for IgG secreting cells

ELISPOT results for total IgG secreting cells in PBMCs

Preliminary assays were carried out to determine the optimum dilutions of capture antibody and the optimum number of cells for an ELISPOT assay to detect total IgG-secreting B cells. Plates were coated with anti-horse IgG (H+L) at dilutions of 1 in 1250 and 1 in 2500 in TBS. Wells were also coated with BSA to control for non-specific binding. PBMCs were diluted twofold from 7.8×10^6 cells/ml to 7.7×10^3 cells/ml and 100μl added to each well. Each dilution of cells was added in duplicate to the wells coated with anti-equine IgG and to wells coated with BSA.

When high numbers of cells were used there was a high background level of staining that appeared as lots of small crystal–like blue dots. Whilst the background staining could be differentiated from true ELISPOTS, it was difficult to determine the number of real ELISPOTs present in these wells. No ELISPOTs were detected at the highest number of cells, 7.8×10^5 cells/well (Table 6.17). The background staining was a problem until the fourth dilution of cells, 9.8×10^4 cells/well, for both dilutions of capture antibody. There was a prozone effect with the number of ELISPOTs detected with both dilutions of capture antibody – the greatest number of ELISPOTs were detected at the fourth dilution of cells (9.8×10^4 cells/well) (Table 6.17). There may have been more ELISPOTs in the wells that had higher numbers of cells but these were not countable due to the background staining.

Non-specific binding of IgG-secreting B cells was not a significant problem; for dilutions of cells from $2x10^5$ to $2.5x10^4$ cells/well, and 6.1×10^3 cells/well, means of less than one ELISPOT per BSA-coated well were detected (Table 6.17).

Table 6.17: ELISPOT for IgG–secreting B cells. Mean number of ELISPOTS detected per well when different numbers of cells added per well. Wells coated with two dilutions of anti-horse IgG and BSA.

Number of cells/well	Mean number of ELISPOTs		
	Anti-horse IgG (1 in 1250)	Anti-horse IgG (1 in 2500)	BSA (0.4mg/ml)
$7.8x10^5$	0	0	0
$3.9x10^5$	5	7	0
$2.0x10^5$	12	7	<1/well
$9.8x10^4$	13	18	<1/well
$4.9x10^4$	7	9	<1/well
$2.5x10^4$	8	6	<1/well
$1.2x10^4$	4	2	0
$6.1x10^3$	1	<1/well	<1/well
$3.1x10^3$	0	2	0
$1.5x10^3$	0	0	0
$7.7x10^2$	0	<1/well	0
No cells	<1/well	0	0

The 1 in 2500 dilution of capture antibody detected the highest number of ELISPOTs, at the dilution of 9.8 x 10^4 cells/well. The numbers of IgG secreting cells/10^5 PBMCs were more consistent between the dilutions of cells from 9.8 x 10^4 to 1.2 x 10^4 cells/well at the 1 in 2500 dilution of capture antibody, than for the same dilutions of cells at the 1 in 1250 dilution of capture antibody (Table 6.18). There was a mean number of 17 (range 12-22) IgG secreting cells/10^5 PBMCs detected at the 1 in 2,500 dilution of capture antibody, and a mean number of 28 (range 13-56) IgG secreting cells/10^5 PBMCs detected at the 1 in 1250 dilution.

Table 6.18: ELISPOT for IgG secreting B cells. Number of IgG-secreting B cells per 10^5 PBMCs, with different dilutions of capture antibody and different numbers of cells.

No. of cells/well	No. of IgG - secreting B cells/10^5 PBMCs	
	Anti-horse IgG (1 in 1250)	Anti-horse IgG (1 in 2500)
9.8x10^4	13	18
4.9x10^4	13	17
2.5x10^4	30	22
1.2x10^4	56	12

The ELISPOT assay was repeated with further titrations of the capture antibody, to ensure optimisation. The ELISPOT plates were coated with anti-horse IgG diluted twofold from 1 in 625 to 1 in 10000. PBMCs isolated from a control horse were used to compare the results with the previous experiment. The cells were added to wells coated with the five dilutions of coating antibody and to wells coated with BSA at 10^5 cells/well and 10^4 cells/well. Cells (5x10^4 cells/well) were also added to wells coated with 1 in 625 to 1 in 2500 dilutions of capture antibody, and those coated with BSA.

ELISPOTs were detected when wells were coated with anti-horse IgG diluted from 1 in 625 to 1 in 2500; very few ELISPOTs were detected at a dilution of 1 in 5000, and none at 1 in 10000 dilution of capture antibody (Table 6.19). At each dilution of capture antibody, the decrease in the number of ELISPOTS detected was not consistent with a decrease in the number of cells/well. The greatest number of ELISPOTs were detected with $5x10^4$ cells/well at dilutions of 1 in 625 and 1 in 1250 capture antibody, and with 10^4 cells/well for the 1 in 2500 dilution. At dilutions of 10^5 cells/well, and $5x10^4$ cells/well there was a decrease in the number of ELISPOTs with increasing dilutions of capture antibody. However with 10^4 cells/well there was an increase in the number of ELISPOTS detected with decreasing dilutions of capture antibody. There was no detection of ELISPOTS in wells coated with BSA.

Table 6.19: ELISPOT for IgG–secreting B cells. Mean number of ELISPOTS detected per well. Wells coated with doubling dilutions of anti-horse IgG capture antibody between 1 in 625 and 1 in 10,000 and BSA (see experiment two for flow cytometry results for these PBMCs).

Capture antibody /coating antigen	Dilution of capture antibody	Mean number of ELISPOTS/well			
		10^5 cells/well	5×10^4 cells/well	10^4 cells/well	No cells
Anti-	**1 in 625**	11	16	1	0
horse	**1 in 1250**	5	6	2	0
IgG	**1 in 2500**	3	0	4	1
	1 in 5000	1	NT[1]	2	NT
	1 in 10000	0	NT	0	NT
BSA		0	0	0	<1

[1] NT = not tested at this dilution

When the results were calculated as number of IgG-secreting cells/10^5 PBMCs, there was variation in the numbers obtained, depending on the original numbers of cells used (Table 6.20). The highest mean number of IgG-secreting cells were detected at the 1 in 625 dilution of capture antibody as 18 (range 10-32) IgG-secreting cells/10^5 PBMCs, followed by the 1 in 2,500 dilution that detected a mean number of 14 (3-40) IgG-secreting cells/10^5 PBMCs. The detection of 14 IgG-secreting cells/10^5 PBMCs is equivalent to only 2% of all B cells as determined by the flow cytometry results for this horse (see experiment two).

Table 6.20: ELISPOT for IgG–secreting B cells. Mean number of IgG-secreting B cells per 10^5 PBMCs when different numbers of PBMCs added per well. Wells coated with doubling dilutions of anti-horse IgG capture antibody between 1 in 625 and 1 in 10000 and BSA (see experiment two for flow cytometry results for these PBMCs).

Dilution of anti-horse IgG	**Number of IgG-secreting cells/10^5 PBMCs**		
	10^5 cells/well	**5 x 10^4 cells/well**	**10^4 cells/well**
1 in 625	11	32	10
1 in 1250	5	12	20
1 in 2500	3	ND[1]	40
1 in 5000	1	NT[2]	20

[1] ND = not determined as no ELISPOTs detected in well

[2] NT = not tested at this dilution of cells

ELISPOT for IgG secreting cells specific for *C. novyi* type A surface antigens and BoNT/C in PBMCs

PBMCs from a horse with CGS were added to an ELISPOT plate coated with *C. novyi* type A surface antigens. The numbers of cells added to the wells were 2.1 x 10^4 cells/well, 1.1 x10^4, 1.1 x 10^3 cells/well and 1.1 x 10^2 cells/well. Some wells received no cells. However, no ELISPOTs were detected.

The experiment was repeated using PBMCs from two other horses with CGS. Plates were coated with BoNT/C as well as *C. novyi* type A surface antigens. PBMCs were added from one horse at 10^5 cells/well, 5 x 10^3 cells/well, and 5 x 10^2 cells/well; the other horse had PBMCs added at 2 x 10^5 cells/well, 10^4 cells/well and 10^3 cells/well. Some wells received no cells. Again, no ELISPOTS were detected in wells coated with the surface antigens or BoNT/C.

ELISPOT for IgG secreting cells from the lamina propria and Peyer's patches of the equine GALT

Preliminary assays were carried out to determine the optimum dilution of capture antibody for an ELISPOT assay to detect IgG secreting B cells isolated from lamina propria or Peyer's patches. An ELISPOT plate was coated with fivefold dilutions of anti-horse IgG antiserum from 1 in 100 to 1 in 12500. Cells were isolated from the lamina propria and Peyer's patches of the GALT of a horse with glomerulonephritis (see experiment four in flow cytometry results). The cells used in the ELISPOT assays were those isolated after the digestion of the relevant tissue with collagenase. Cells isolated from the lamina propria were added to wells at dilutions of 2 x 10^5 cells/well and 10^5 cells/well, and cells isolated from the Peyer's patches were added to the wells at a dilution of 2 x10^5 cells/well. Cells were also added to

wells coated with *C. novyi* type A surface antigens, *C. botulinum* type C surface antigens, BoNT/C and BSA. Cells were added in triplicate.

When wells were coated with anti-equine IgG at dilutions of 1 in 100 to 1 in 2500, the numbers of ELISPOTS produced were too many to count accurately when either 2×10^5 cells/well or 10^5 cells/well cells from the lamina propria or Peyer's patches were used. However, when wells were coated with the 1 in 12500 dilution of anti-equine IgG ELISPOTS were only detected with cells isolated from the Peyer's patch; a mean of three ELISPOTS were detected per well (Table 6.21). These results suggest that the cells need to be titrated out further and added to wells coated with at least 1 in 2500 dilution of capture antibody.

A mean number of two ELISPOTs per well were counted to *C. novyi* type A surface antigens when 2×10^5 cells/well were added from the lamina propria or Peyer's patch. No ELISPOTs were detected to BSA or BoNT/C. A mean of less than one ELISPOT per well was detected to the surface antigens of *C. botulinum* type C.

Table 6.21: ELISPOT for IgG–secreting B cells. (a) Mean number of ELISPOTS detected per well when different numbers of lamina propria and Peyer's patches cells were added per well. Wells were coated with fivefold dilutions of anti-horse IgG capture antibody between 1 in 100 and 1 in 12500, *C. novyi* type A surface antigens, *C. botulinum* type C surface antigens, BoNT/C and BSA.

Capture antibody/ coating antigen	**Mean number of ELISPOTS per well**			
	Cells isolated from lamina propria (2×10^5 cells/well)	**Cells isolated from lamina propria (10^5 cells/well)**	**Cells isolated from Peyer's patch (2×10^5 cells/well)**	**No cells**
Anti-equine IgG (1 in 100)	>30	>30	>30	0
Anti-equine IgG (1 in 500)	>30	>30	>30	0
Anti-equine IgG (1 in 2500)	>30	>30	>30	0
Anti-equine IgG (1 in 12500)	<1	0	3	0
***C. novyi* type A**	2	0	2	0
***C. botulinum* type C**	<1	<1	0	0
BoNT/C	0	0	0	0
BSA	0	0	0	0

When results were expressed as mean number of IgG secreting cells/10^5 cells, only two IgG-secreting cells per 10^5 Peyer's patch cells were detected at the 1 in 12500 dilution of capture antibody (Table 6.22). This is equivalent to 0.17% of the B cells as determined by the flow cytometry results (see experiment four). However, the capture antibody diluted 1 in 12500 is below the optimum for this assay and therefore this percentage is probably not a true reflection of the detection abilities of this assay. The number of IgG secreting cells could not be calculated for the other dilutions of capture antibody as there were too many ELISPOTs to count. One anti-*novyi* IgG secreting cell per 10^5 Peyer's patch cells and one anti-*novyi* IgG secreting cell per 10^5 lamina propria cells were detected. This is equivalent to 0.08% of the B cells in the Peyer's patch and 0.07% of B cells in the lamina propria, being specific for *C. novyi* type A. When a mean of less than one ELISPOT was detected per well, the number of IgG secreting cells per 10^5 cells could not be calculated.

Table 6.22: ELISPOT for IgG–secreting B cells. Mean number of IgG-secreting B cells per 10^5 cells isolated from GALT when different numbers of lamina propria and Peyer's patches cells added per well. Wells were coated with fivefold dilutions of anti-horse IgG capture antibody between 1 in 100 and 1 in 12500, *C. novyi* type A surface antigens, *C. botulinum* type C surface antigens, BoNT/C and BSA.

Capture antibody/ coating antigen	**Number of IgG-secreting cells/10^5 GALT cells**		
	Lamina propria ($2x10^5$ cells/well)	**Lamina propria (10^5 cells/well)**	**Peyer's patch ($2x10^5$ cells/well)**
Anti-equine IgG (1 in 12500)	ND[1]	ND	2
***C. novyi* type A surface antigens**	1	ND	1

[1]ND: not determined as a mean of less than one ELISPOT per well was detected

The assay was repeated with cells isolated from the lamina propria of the GALT from a horse initially clinically diagnosed with AGS, but not confirmed by histopathology at post-mortem. The subsequent diagnosis was thought to be botulism. The cells used in the assay were the cells released by collagenase digestion of the lamina propria, the cells at the interface after separation of these cells on Histopaque, and PBMCs from the same horse. Wells were coated with 1 in 2500 dilution of anti-equine IgG antibody. Cells from the lamina propria were added to the wells in triplicate at numbers of 10^6, 10^5 and 10^4 cells per well; cells form the lamina propria separated on Histopaque were added at $5x10^5$, $5x10^4$ and $5x10^3$ cells/well; and PBMCs were added at 10^4, $5x10^3$ and 10^3 cells/well. Cells were also added to wells coated with *C. novyi* type A surface antigens, BoNT/C and BSA, but only at 10^6 lamina propria cells/well, $5x10^5$ lamina propria cells separated on Histopaque/well and 10^4 PBMCs/well. Cells were added to wells coated with *C. botulinum* type C at 10^6 and 10^5 lamina propria cells/well, $5x10^5$, $5x10^4$ lamina propria cells separated on Histopaque/well, and 10^4, $5x10^3$ PBMCs/well.

For cells isolated from the lamina propria, including those separated by Histopaque, when 10^6 or $5x10^5$ cells/well were used there were too many ELISPOTS for total IgG secreting cells to count. However 10^5 cells/well or less produced countable numbers of ELISPOTs. 10^4 and $5x10^3$ PBMCs/well produced countable numbers of ELISPOTs in wells coated with anti-equine IgG antibodies (Table 6.23). There was a noticeable decrease in background staining with decreasing numbers of cells. ELISPOTs were detected in wells coated with *C. novyi* and *C. botulinum* surface antigens when cells from the lamina propria were added. However, cells from the lamina propria also secreted IgG to BSA. The number of ELISPOTs in wells coated with *C. botulinum* type C surface antigens was greater than the numbers of ELISPOTs in wells coated with BSA for all three cell groups.

Table 6.23: ELISPOT for IgG–secreting B cells. Mean number of ELISPOTS detected per well when different numbers of lamina propria cells added per well. Wells were coated with anti-horse IgG capture antibody, *C. novyi* type A surface antigens, *C. botulinum* type C surface antigens, BoNT/C and BSA.

		Mean number of ELISPOTS per well				
		Capture antibody/ coating antigen				
Cells	**No. of cells/ Well**	**Anti-equine IgG (1 in 2,500)**	***C. novyi* type A surface antigens**	***C. botulinum* type C surface antigens**	**BoNT/C**	**BSA**
Lamina propria cells	**10^6**	>50	11	13	8	11
	10^5	24	NT[1]	3	NT	NT
	10^4	5	NT	NT	NT	NT
Lamina propria cells (Histopaque)	**$5x10^5$**	>50	6	14	5	6
	$5x10^4$	23	NT	1	NT	NT
	$5x10^3$	1	NT	NT	NT	NT
PBMCs	**10^4**	9	0	<1	0	0
	$5x10^3$	4	NT	0	NT	
	10^3	<1	NT	NT	NT	
No cells	**0**	0	0	0	0	0

[1] NT= not tested

When results were expressed as the number of IgG-secreting cells B cells per 10^5 isolated cells there was some variation in these numbers when calculated for the different numbers of cells added initially (Table 6.24). A mean of 37 total IgG-secreting cells/10^5 lamina propria cells were detected compared to a mean of 33 total IgG-secreting cells when the lamina propria cells were separated on Histopaque. A mean of 85 total IgG-secreting cells/10^5 PBMCs were detected, a greater number than from the lamina propria. A mean of two anti-*C. botulinum* type C IgG-secreting cells/10^5 lamina propria cells were detected, compared to a mean of three anti-*C. botulinum* type C IgG-secreting cells when the lamina propria cells were separated on Histopaque. These results are equivalent to the detection of 5% of B cells isolated from the lamina propria, and 0.3% of the isolated B cells are specific for *C. botulinum* type C (flow cytometry results not shown for this horse). Another way of expressing these results is that 5% of IgG-secreting B cells in this assay were specific for *C. botulinum* type C.

Table 6.24: ELISPOT for IgG–secreting B cells. Mean number of IgG-secreting B cells per 10^5 cells isolated from GALT when different numbers of lamina propria cells added per well. Wells coated with anti-horse IgG capture antibody, *C. novyi* type A surface antigens, *C. botulinum* type C surface antigens, BoNT/C and BSA.

		Number of IgG-secreting B cells per 10^5 isolated cells				
		Capture antibody/ coating antigen				
Cells	**No. of cells/ Well**	**Anti-equine IgG (1 in 2500)**	***C. novyi* type A surface antigens**	***C. botulinum* type C surface antigens**	**BoNT/C**	**BSA**
Lamina propria cells	**10^6**	ND[1]	1	1	1	1
	10^5	24	NT[3]	3	NT	NT
	10^4	50	NT	NT	NT	NT
Lamina propria cells (Histopaque)	**$5x10^5$**	ND[1]	1	3	1	1
	$5x10^4$	46	NT	2	NT	NT
	$5x10^3$	20	NT	NT	NT	NT
PBMCs	**10^4**	90	ND[2]	ND[2]	ND[2]	ND[2]
	$5x10^3$	80	NT	ND[2]	NT	NT
	10^3	<20	NT	NT	NT	NT

[1] ND = not determined as more than 50 ELISPOTS counted in original well
[2] ND = not determined as less than one ELISPOT counted per well
[3] NT = Not tested

ELISPOT for IgA secreting cells from the lamina propria and Peyer's patches of the equine mucosa

Preliminary assays were carried out to determine the optimum dilution of capture antibody for an ELISPOT assay to detect IgA-secreting B cells. An ELISPOT plate was coated with tenfold dilutions of anti-equine IgA mouse monoclonal (BVS) from 1 in 100 to 1 in 100000. Cells were isolated from the lamina propria and Peyer's patch of the GALT of a horse with glomerulonephritis. Cells were added to the wells in triplicate to give 2 x 10^5 cells/well and 10^5 lamina propria cells/well, and 10^5 Peyer's patch cells/well. Cells were also added to wells coated with *C. novyi* type A surface antigens, *C. botulinum* type C surface antigens, BoNT/C and BSA. However, no ELISPOTs were detected.

The ELISPOT was repeated using PBMCs and cells isolated from the lamina propria from the horse diagnosed as query botulism. Wells were coated with anti-equine IgA at dilutions of 1 in 1000 and 1 in 10000. Cells were added in triplicate to wells coated with anti-equine IgA diluted 1 in 1000 to give 10^6, 10^5 and 10^4 lamina propria cells/well, $5x10^5$, $5x10^4$ and $5x10^3$ lamina propria cells (after separation on Histopaque)/well; and 10^4, $5x10^3$ and 10^3 PBMCs /well. Wells coated with anti-equine IgA diluted 1 in 10000, *C. novyi* type A, *C. botulinum* type C, BoNT/C and BSA received 10^6 lamina propria cells/well, $5x10^5$ lamina propria cells (after separation on Histopaque)/well, and 10^4 PBMCs/well. However, again there were no ELISPOTs detected. There was a great deal of background staining, particularly when cells were coated with anti-equine IgA. This made it very difficult to determine whether there were true ELISPOTs present.

6.1.5. Detection of antibodies in saponin-extracted GALT

ELISA for total IgG in GALT extracts

Optimisation of dilutions for assay

Samples of tissue from Peyer's patches or lamina propria were treated overnight with saponin and the supernatant assayed for antibody content. Preliminary assays were carried out to identify the appropriate dilution range for the screening of extracts for calculation of total IgG content in the GALT extracts. Extracts from the lamina propria and Peyer's patches from two horses were diluted tenfold between 1 in 5 and 1 in 500000. The IgG content in the extracts of lamina propria and Peyer's patches from each horse were very similar, but there was a difference in IgG content between horses (Fig. 6.14a).

Faecal and ileum content extracts, treated with protease inhibitors (Chapter 2.3.2), from three horses were also assayed for total IgG content. Samples were diluted tenfold between 1 in 5 and 1 in 500000. There was some variation in IgG content both between horses and between samples. A prozonal effect was observed up to the 1 in 50 dilution with faecal sample 2 and ileum contents 2 and up to the 1 in 500 dilution with ileum contents 3 (Fig. 6.14b). However, the other samples showed a decrease in IgG content from the 1 in 5 dilution.

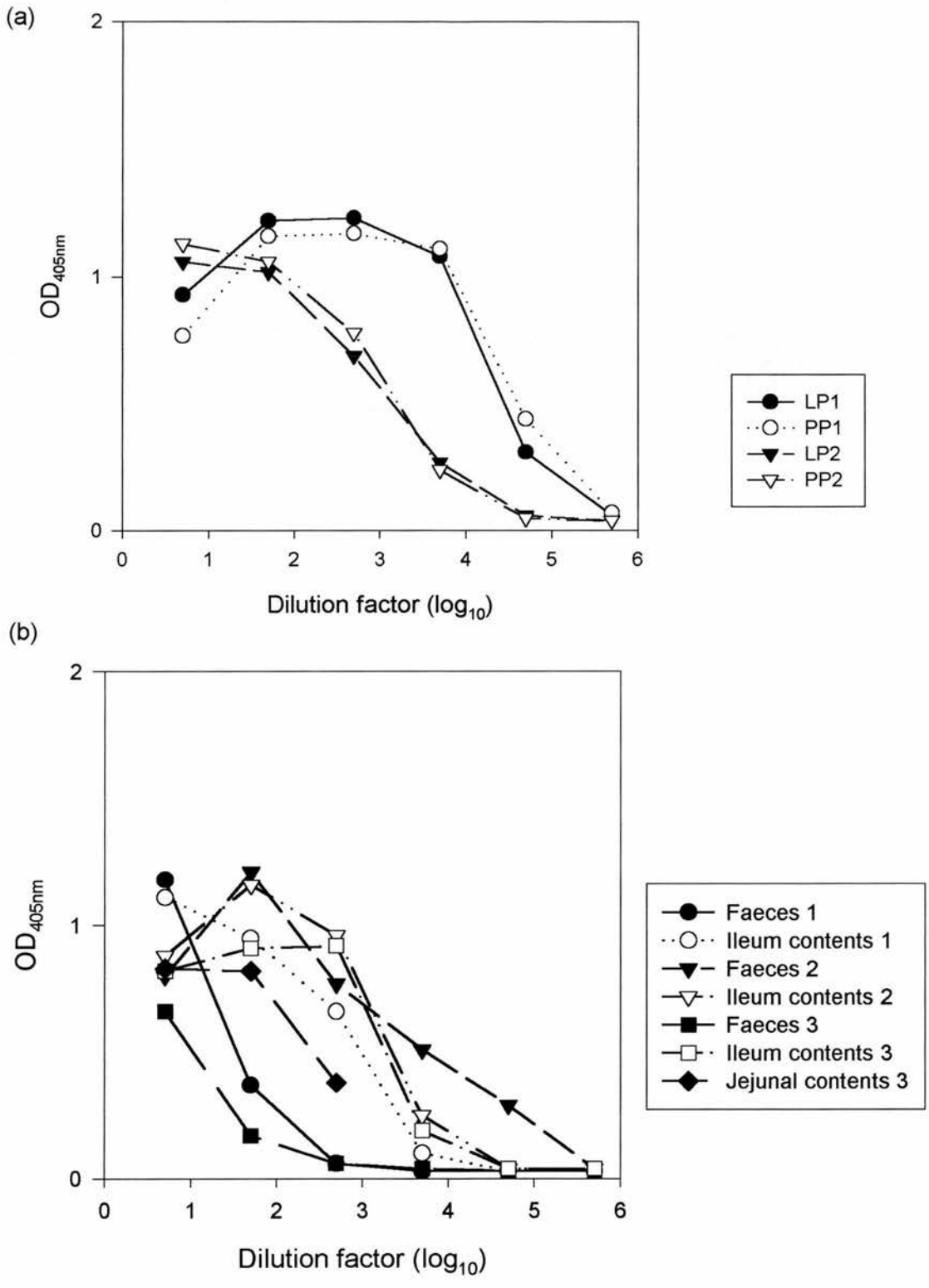


Figure 6.14: ELISA for total IgG in (a) saponin-digested GALT extracts from two different horses (1 and 2) and (b) protease inhibitor treated faeces and intestinal contents from three different horses (1, 2 and 3). Samples diluted tenfold from 1 in 5 to 1 in 500,000.
LP= lamina propria
PP= Peyer's patches

On the basis of these results, GALT extracts should be titrated tenfold between 1 in 50 and 1 in 50000 for further screening of samples to determine total IgG content; faecal and ileum contents should be titrated tenfold between 1 in 5 and 1 in 5000. This should enable calculation of IgG content based on a dilution falling on the linear part of the dilution curve for individual samples.

Optimisation of length of incubation with saponin

Preliminary assays were carried out to investigate the effect of incubation time with saponin on antibody recovery in the extract. Tissue samples from the lamina propria were treated with saponin for different incubation lengths – 2 h, 4 h, 6 h, 8 h and 30 h. The extracts were diluted fivefold between 1 in 1000 to 1 in 125000 and assayed for total IgG content by ELISA.

It was found that the longer the incubation length with saponin, the greater the level of IgG antibody in the tissue extract; this was the case for up to 30 h (Fig. 6.15). There was little difference in the IgG content of the extract between 4 and 8h incubation. In previous studies, tissue samples had been thawed overnight in the presence of saponin (Bergquist et al, 2000; Bergquist et al, 1995). All further samples were incubated overnight with saponin.

Total IgG content in GALT extracts

Saponin extraction of the tissue from the lamina propria and Peyer's patches resulted in a ten-fold increase in total IgG compared to the protease inhibitor-treated ileum contents, and a 100-fold increase in IgG compared to the faeces (Table 6.25). However, these results are based on only a few samples.

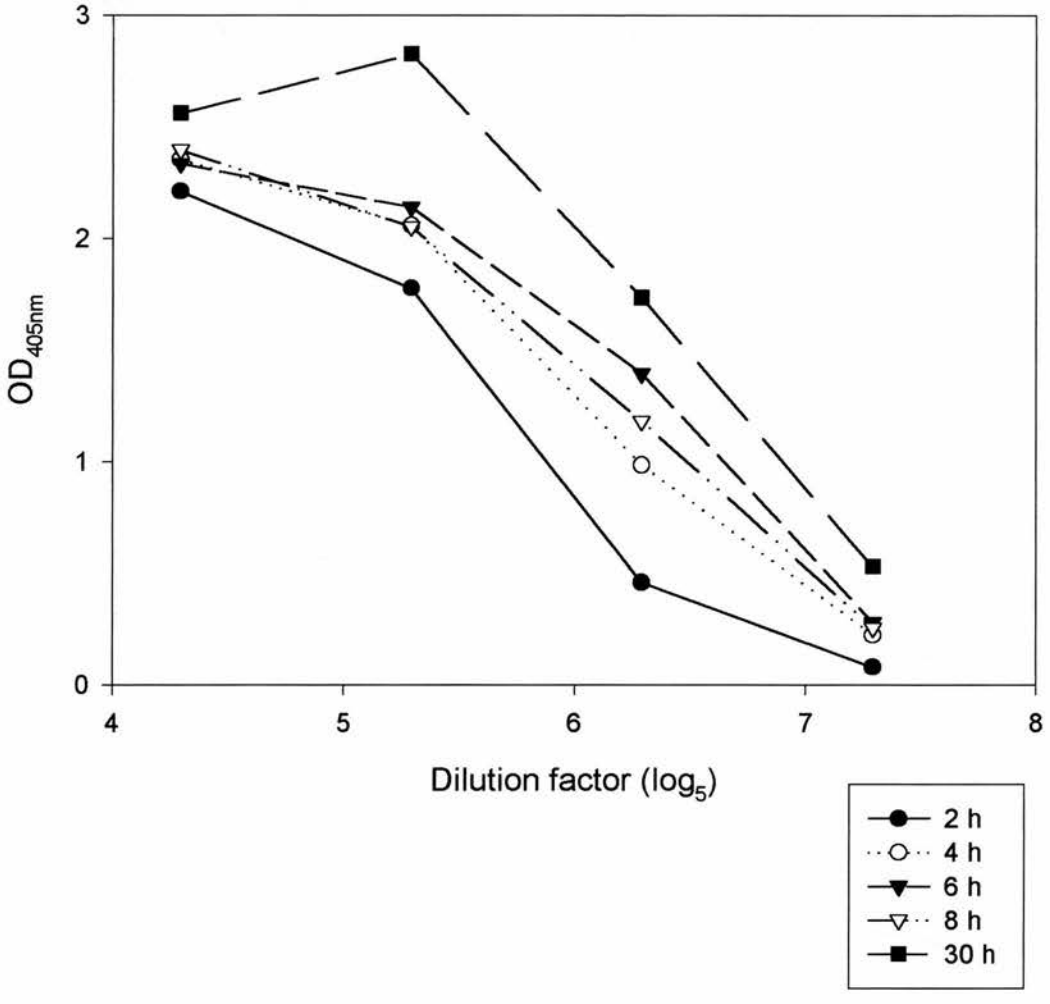


Figure 6.15: Total IgG in lamina propria extracts incubated for 2 h, 4 h, 6 h, 8 h, and 30 h with saponin. Extracts diluted fivefold between 1 in 1,000 and 1 in 125,000.

Table 6.25: Total IgG in saponin extracts from the lamina propria and Peyer's patches, and in protease treated faeces, ileum contents and jejunal contents.

Statistics	Total IgG mg/ml				
	Lamina propria (n=2)	**Peyer's patches (n=2)**	**Ileum contents (n=3)**	**Faeces (n=3)**	**Jejunum contents (n=1)**
Mean	1.07	1.42	0.11	0.014	0.02
SD	1.46	1.95	0.06	0.02	N/A[1]
Range	0.04-2.1	0.04-2.8	0.04-0.15	0.001-0.04	N/A

[1] N/A = not applicable as only one sample in group

ELISA for total IgA in GALT extracts

Optimisation of assay

Preliminary assays were carried out to determine the dilution range suitable for assaying tissue extracts for total IgA. Saponin extracts, from the lamina propria and Peyer's patches of two horses, were diluted tenfold between 1 in 5 and 1 in 500000. However, IgA could only be detected at the lowest dilution (1 in 5) in the extracts from the lamina propria and Peyer's of horse two (Fig. 6.16a); horse two had a lower total IgG content in these extracts compared to horse one (Fig. 6.14a). The OD measurements for IgA in both the lamina propria and Peyer's patches extracts were below the negative control for horse one, even at the 1 in 5 dilution.

The highest OD reading was obtained for the protease treated faecal sample two (Fig. 6.16b). Ileum contents two and three, faecal sample three and jejunal contents three, all had OD measurements above the negative control at the 1 in 5

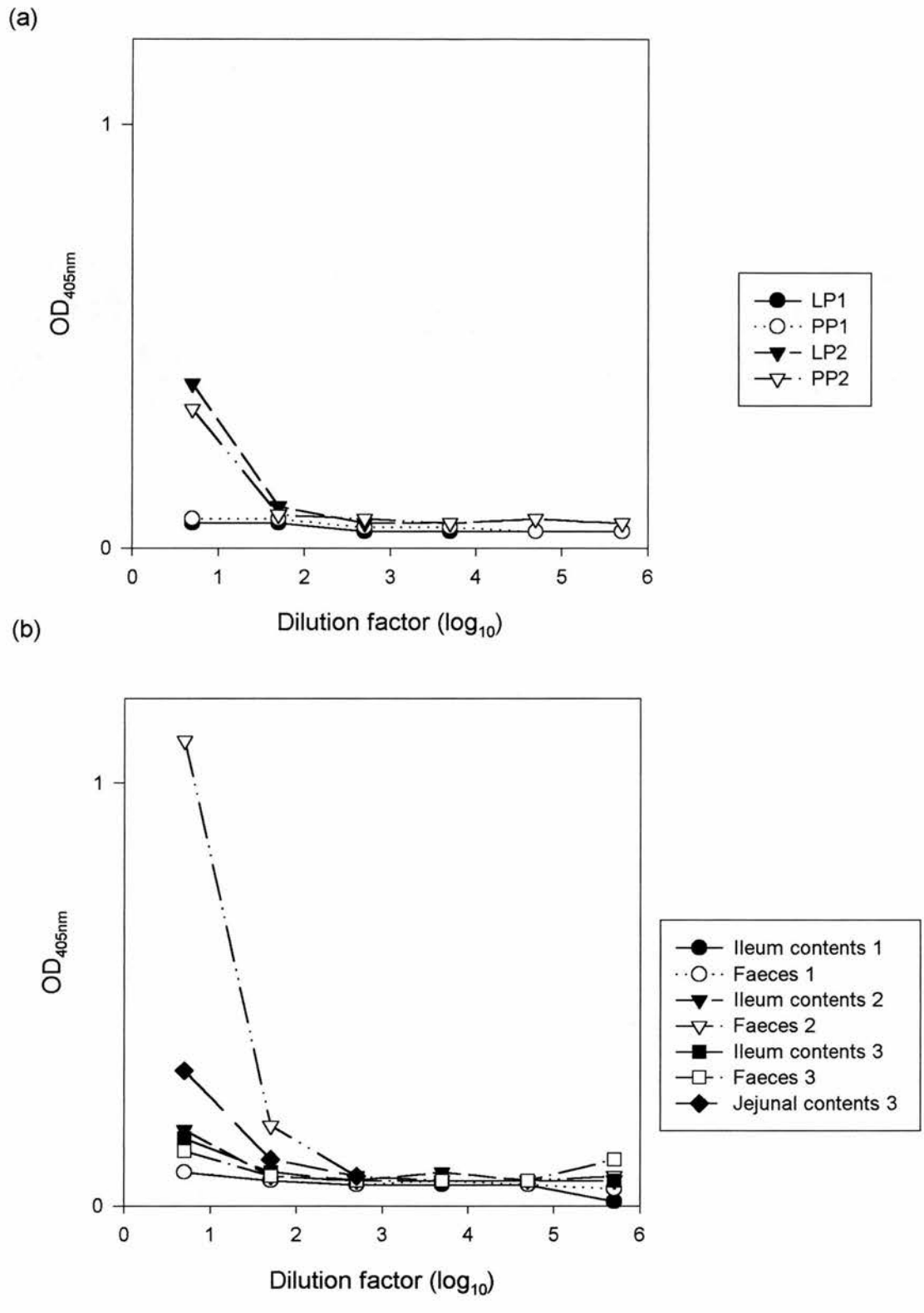


Figure 6.16: IgA in (a) saponin extracts of lamina propria and Peyer's patches from two different horses and (b) protease inhibitor treated faeces and ileum contents from three different horses. Samples were diluted tenfold between 1 in 5 and 1 in 500,000. LP=lamina propria; PP=Peyer's patches.

dilution. However, only faecal sample two had an OD level above the negative control at the 1 in 50 dilution.

Calculation of total IgA

It was not possible to calculate the total IgA content in these samples by ELISA due to failure of the IgA standard. However, the total IgA content of both extracts from the lamina propria and Peyer's patches from horse two (LP2 and PP2) were determined by RID to be 1.5mg/ml.

6.2 Discussion

6.2.1 Optimisation of ELISA to detect IgA to *C. novyi* type A surface antigens and BoNT/C in GI contents

Several problems were encountered in the development of an ELISA to detect specific IgA in samples from the equine GI tract. The most significant initial problem was the lack of a known positive control for either the assay for IgA to the surface antigens or to BoNT/C. At the time of this study, it was not possible to determine total IgA content in equine faecal and ileum samples – attempts to develop an ELISA for total IgA with available reagents had been unsuccessful. Therefore it was not possible to assess if the treatment of samples, for inhibition of intestinal proteases, was effective in preserving intestinal IgA.

Initial assays, used *C. novyi* type A surface antigen coated plates. Previous work had shown that the majority of horses had high levels of serum IgG to the surface

antigens (Chapter five). On this basis it was initially thought that the majority of horses might also have intestinal IgA to the surface antigens. EDTA-extracted antigens were also readily available, unlike purified BoNT/C, and therefore it was considered more economical to do the initial assays using the surface antigen coated plates. Previous work also had shown the presence of *C. botulinum* type C in the GI tract of horses with grass sickness (Chapter three). Therefore initial assays were done using samples from horses with chronic grass sickness, on the basis that they were more likely to have been exposed to the organism and to have made a specific intestinal IgA response to it.

Initial incubation times for the ELISA were lengthy – overnight with sample, five hours with primary detecting antibody, overnight with conjugate – in order to increase the chances of detection of specific IgA. Samples were identified that gave positive OD readings in this ELISA, allowing optimisation of the assay with respect to dilution of reagents. However, attempts to shorten the incubation times to make the ELISA more time-effective resulted in a significant decrease in OD measurements, and initial incubation lengths were kept.

In retrospect, the development of the assay may have been less time consuming if BoNT/C coated plates had been used, as it was found that higher levels of IgA to BoNT/C than IgA to the surface antigens were usually detected in faeces and ileum contents.

6.2.2 Detection of specific IgA to *C. botulinum* type C in equine GI tract

IgA, both to surface antigens and BoNT/C, was detected in the ileum and/or the faeces of a higher percentage of horses with grass sickness compared to controls. Horses with grass sickness also had a statistically significant higher level of IgA to BoNT/C in their faeces compared to controls. These results suggest that horses with grass sickness had been exposed to BoNT/C in the GI tract, resulting in the production of specific IgA to the toxin. As the mucosal immune response is thought to be relatively short-lived (Pierce and Cray, 1982), detection of IgA to BoNT/C in the GI tract also suggests that horses with grass sickness had recently been exposed to BoNT/C.

When the horses with grass sickness were grouped by disease category, only horses with the subacute form of the disease had significantly higher levels of IgA to BoNT/C and to the surface antigens in the faeces, compared to the control horses. Horses with subacute grass sickness also had significantly higher levels of IgA to BoNT/C in the ileum contents compared to controls. Horses with subacute grass sickness had detectable IgA to BoNT/C and surface antigens in the highest percentage of faecal samples, and IgA to BoNT/C in the highest percentage of ileum samples, compared to the other groups of horses. Differences in duration of exposure to toxin and size of toxin dose may explain differences in antibody levels detected in the different clinical categories of equine grass sickness. Grass sickness is a spectrum of disease that has been divided up into three categories. The clinical severity of grass sickness is associated with the extent of neuronal damage (Scholes et al, 1993a). It has been hypothesised that the extent of neuronal damage reflects the size of the toxic insult (Doxey et al, 1995a): acute grass sickness would be associated with a large amount of toxin, and chronic grass

sickness a much smaller amount of toxin. The duration of the disease is also governed by the extent of neuronal damage; less neuronal damage results in less severe disease and longer survival time.

Hypothetically, horses with subacute grass sickness would be exposed to an intermediate amount of toxin in comparison to horses with acute and chronic grass sickness. The neuronal damage is less than in acute grass sickness and horses with the subacute form can survive for up to seven days. The amount of toxin in the gut may be sufficient to stimulate a mucosal immune response, and the longer duration is sufficient to detect the production of specific IgA. Although horses with acute grass sickness are probably exposed to large doses of toxin, they die or are euthanased within 48 hours of onset of clinical symptoms. The short duration of disease may account for the lower levels of IgA detected in horses with acute grass sickness.

However, the detection of IgA in the gut of some of horses with acute grass sickness is particularly interesting, as it suggests that these horses were exposed to BoNT/C prior to the onset of clinical symptoms. BoNT/C has been detected in the GI tract of horses with grass sickness (Chapter 3), but it has not yet been demonstrated to be the cause of the disease. GI stasis is a major clinical finding in equine grass sickness, and it is possible that overgrowth of *C. botulinum* type C into the ileum with subsequent production of BoNT/C occurs as a result of GI stasis rather than causing it. However, if BoNT/C was produced after the onset of GI stasis, it would be unlikely that specific IgA to BoNT/C could be detected in horses with acute grass sickness, theoretically within 24-48h of exposure to the toxin. Therefore, detection of IgA to BoNT/C in the GI tract of horses with acute grass sickness may be

evidence in support of the hypothesis that *C. botulinum* type C is the cause of equine grass sickness.

The only significant difference between the groups of grass sickness horses, were in IgA levels to BoNT/C in the faeces of horses with subacute grass sickness and horses with chronic grass sickness. This may reflect exposure to a smaller dose of toxin in horses with chronic grass sickness, resulting in production of lower levels of specific antibodies. However, the differences in antibody levels may also reflect the point in the disease at which the faecal sample was collected. Fluctuation in IgA both to surface antigens and to BoNT/C was observed in the faeces of horses with CGS that were sampled more than once. These fluctuations in specific IgA levels between faecal samples may be evidence of a mucosal immune response being made to the antigens, as well as neutralisation of the antigens by antibody. Decreases in OD may be due to the inability of the ELISA to detect antibody bound to specific antigen. With the EndoCAb ELISA, an ELISA which measures antibodies specific for the endotoxin core, decreases in antibody levels to endotoxin are seen in patients exposed to systemic endotoxin (Barclay, 1995). The observed changes in IgA levels over the course of grass sickness were relatively small changes in OD measurements, and therefore it is not known whether they are significant in vivo.

There is insufficient evidence to state whether specific IgA to these antigens are important in the recovery of horses from chronic grass sickness. In horses that were sampled more than once, recovery was not always associated with an increase in faecal IgA to the surface antigens and toxin. Horses that recovered from chronic grass sickness had a higher mean level of IgA both to the surface antigens and toxin at the start and end of the disease compared to horses that were euthanased, but this difference was not statistically significant.

Theoretically, the presence of IgA to BoNT/C would be considered to be beneficial to the horse as it would limit exposure to the toxin, preventing or limiting the severity of the disease. However, the detection of IgA to BoNT/C in this study in horses with grass sickness that were euthanased, would not appear to support a positive role for mucosal IgA. It may be that these horses did not have a primed mucosal immune response to BoNT/C – the IgA detected in this study may be the result of a primary mucosal immune response, the production of which was too slow to prevent toxin damage. Horses with grass sickness have been shown to have lower systemic IgG both to surface antigens and BoNT/C compared to controls (Chapter 5). Whilst the control horses in this study had lower levels of IgA to BoNT/C in the faeces than horses with grass sickness, this may be due to the fact that they have not been recently exposed to the organism. The control horses may have good mucosal memory responses to BoNT/C. The detection of IgA to BoNT/C in the faeces of some control horses may reflect subclinical cases of grass sickness. Subclinical cases of grass sickness are thought to occur, although they have not been proven. Investigation of faeces from horses co-grazing with cases of grass sickness, for the presence of IgA to BoNT/C would help to identify a role for mucosal IgA in the prevention of grass sickness.

Changes in specific IgA both to BoNT/C and surface antigens were observed in the faeces of six healthy control horses. Whilst these changes may reflect exposure to the organism and toxin, they are only small differences in OD measurements and may not be significant in vivo. The differences in OD between faecal samples from these healthy horses were generally smaller than those changes observed between consecutive faecal samples from horses with chronic grass sickness.

Only faecal samples from horses with SGS had significantly higher levels of IgA to the surface antigens compared to the control horses. The mean IgA levels to the surface antigens were lower than the mean IgA levels to BoNT/C in both the faeces and ileum contents of all the groups of horses. This is an interesting contrast to the results for systemic IgG to these antigens, where higher levels of IgG were detected to the surface antigens than to BoNT/C (Chapter 5). Although differences in ELISA results do not necessarily reflect real difference in vivo, the same antigens were used in both assays for systemic and mucosal responses. *C. novyi* type A is ubiquitous in the environment (Smith, 1975a) and may normally be present in the GI tract of horses. It is possible that there is a degree of immune tolerance to *C. novyi* type A in the GI tract of the horse due to its common presence; this may account for the low levels of specific IgA detected to the surface antigens in the GI tract. *C. novyi* type A has been used as a surrogate marker for the surface antigens *of C. botulinum* type C in this study. However, results of poor correlation between IgA to *C. novyi* type A surface antigens and IgA to *C. botulinum* type C surface antigens in milk, suggested that *C. novyi* type A might not be as suitable a surrogate marker for the investigation of local immune responses (Chapter 7.1.6). This may be due to immune tolerance to *C. novyi* and therefore also immune tolerance to some of the shared epitopes of *C. botulinum* type C. The local immune response may be more organism-specific than the systemic immune response due to mechanisms of immune tolerance to commensal organisms.

This study has identified higher levels of IgA to BoNT/C in the GI tract of horses with grass sickness compared to controls. However, there are several problems associated with this study. Faecal and ileum samples from different horses may have different intestinal protease activity. The breakdown of IgA in samples could not be monitored due to the lack of an assay for total IgA at the time of the study.

Differences in consistency of samples between horses may also effect results, as wet weight of sample was used in calculating dilution factors. Horses with acute and subacute grass sickness often have liquid stomach and ileum contents due to fluid accumulation. It would probably be expected that this would result in lower OD measurements for these samples due to increased dilution. Although horses with subacute grass sickness had significantly higher levels of specific IgA in this study, the levels produced may indeed have been higher than those detected. Another consideration might be that there may be movement of fluid, out of the intestinal wall into the lumen, leaching high levels of mucosal antibodies into the gut. In contrast to horses with acute or subacute grass sickness, the GI tract of a horse with the chronic form, can be empty of contents at post-mortem, and faeces may be irregularly produced over the course of the disease. An investigation of the mucosal immune response to the specific antigens at the cellular level would eliminate problems associated with protease breakdown of IgA and variation in sample consistency.

6.2.3 Investigation of the GI mucosal immune response at the cellular level

To investigate the mucosal immune response to *C. botulinum* type C at a cellular level, a method for the isolation of functionally active lymphocytes from the equine gut associated lymphoid tissue (GALT) had to be developed. A method for the isolation of lymphocytes from GALT that had been developed and successfully used in the pig (M. Bailey and K. Haverson, personal communication) was adapted for use in the horse. There was no available published data for leucocyte populations in the equine mucosa, and to ensure that lymphocytes could also be isolated from the horse by this method, cells were analysed by flow cytometry. Prior to analysis

of the isolated GALT cells, equine PBMCs were analysed by flow cytometry to identify equine leucocyte populations by bitmap, check specificity of primary antibodies (specific for CD3, surface immunoglobulin and CD18), and optimise conditions for labelling the cells.

Flow cytometric analysis of equine PBMCs

There are few commercially available anti-equine antibodies for use in flow cytometry. However, there have been three international equine leucocyte antigen workshops to date (ELAW I, II and III), and the results of ELAW I and II have been published (Kydd et al, 1991; Lunn et al, 1998). These workshops have identified and evaluated antibodies recognising equine leucocyte antigens, and many of these are available on request from the respective researchers.

The anti-equine CD3 monoclonal antibody used in these experiments recognises the equine equivalent of the CD3 antigen present on all T cells (Lunn et al, 1998). In experiments one and two, the anti-CD3 antibody positively labelled 67-84% of cells in the lymphocyte population. These results are comparable with the results from ELAW II when this antibody was shown to label positively a mean of 75% (range 54-92%) of peripheral blood leucocytes (Lunn et al, 1998). The CVS 36 monoclonal antibody is specific for all isotypes of equine immunoglobulin (Lunn et al, 1998), and therefore can bind to B cells through their expression of surface immunoglobulin (sIg). CVS 36 positively labelled between 12 and 37.4% of cells in the lymphocyte populations in experiments one and two. These results are also comparable with the results from ELAW II, where CVS 36 was shown to label 12% of lymphocytes and 100% of B lymphocytes (defined as CD5-ve lymphocytes) (Lunn et al, 1998).

The anti-CD18 antibody positively labelled 77-95% of cells in the lymphocyte population and 91-99% of cells in the monocyte population of the PBMCs analysed in experiments one and two. The anti-CD18 monoclonal antibody used in this study was an anti-human CD18. CD18 is present on the surface of all leucocytes in conjunction with CD11. The anti-CD18 antibody was not included in the ELAW workshops but had been shown to cross-react with equine leucocytes, amongst other animal species; the antibody had been shown to bind to 100% of equine lymphocytes, monocytes and granulocytes (Jacobsen et al, 1993).

A high percentage of cells in the monocyte population (as determined by the bitmap) in experiment one, were also positively labelled with anti-CD3 and CVS 36. This indicated either inappropriate gating of leucocyte populations by bitmap, or non-specific binding of reagents. The inclusion of a blocking step was successful in reducing the percentage of positively labelled cells in the monocyte population and increasing the percentage of positively labelled cells in the lymphocyte population, but only by a few percent. Even after blocking, CVS 36 still positively labelled a higher percentage of cells in the monocyte population than the lymphocyte population. However, in terms of numbers, there were a greater number of cells in the lymphocyte population positively labelled with CVS 36 than in the monocyte population.

Binding of CVS 36 to cells in the monocyte population may be due to Fc receptor (FcR) binding of equine immunoglobulin by monocytes, with FcR-bound immunoglobulin being detected by the CVS 36 (anti-immunoglobulin) antibody. At ELAW II, monocytes were removed from peripheral blood leucocytes before FACS analysis with anti-immunoglobulin reagents. B cells were pre-incubated in an equine serum–free medium for at least eight hours prior to FACS analysis to ensure

turnover of Fc receptors and removal of any equine immunoglobulin bound to the Fc receptors (Lunn et al, 1998). It was found that if B cells were not pre-incubated or were incubated for less than eight hours, the presence of immunoglobulins bound to Fc receptors resulted in the detection of a variety of different isotype-producing B cells by antibodies specific for only one isotype: the antibodies were binding FcR bound immunoglobulins as well as the sIg. As CVS 36 can detect all immunoglobulin isotypes anyway, pre-incubation to ensure B cell Fc receptor turnover was not really necessary in this study. However, the monocytes had not been removed from the PBMCs and therefore the Fc receptor binding of equine immunoglobulin by monoctyes may be responsible for some binding of CVS 36 to these cells. The monocytes may also be non-specifically binding the anti-CD3 antibody through their Fc receptors, accounting for the CD3 positively labelled cells in the monocyte population. The conjugate is the $F(ab')_2$ fragment of antibody to prevent non-specific binding of the conjugate via Fc receptors; the negative controls demonstrated that there was no binding of the conjugate to cells in the absence of primary antibody.

Isolation of lymphocytes from equine GALT

Initially it was decided to isolate cells from the Peyer's patches as this is where the effector and memory responses are generated in the GALT. GALT can be divided into organised (O-MALT) and diffuse mucosal-associated lymphoid tissue (D-MALT). The O-MALT consists of single lymphoid follicles distributed along the length of the GI tract, Peyer's patches in the small intestine (aggregates of lymphoid follicles) and the mesenteric lymph nodes that drain the mucous membranes. The D-MALT consists of lymphocytes, plasma cells and antigen presenting cells (APC) in the lamina propria, interstitial tissue of the mucosa and glands, and epithelia.

Cells activated in the O-MALT recirculate to the D-MALT where they carry out their effector functions.

Lymphoid follicles and Peyer's patches in the O-MALT are covered by follicle associated epithelium, composed of the normal columnar enterocytes and specialised membranous epithelial cells (M cells) which are adapted for antigen sampling of the luminal contents. Luminal-derived antigens are presented by antigen presenting cells (APC) in the O-MALT and activated lymphocytes preferentially recirculate to the lamina propria of the D-MALT. This recirculation is possibly due, in part, to the up-regulation in surface expression of $\alpha 4\beta 7$ that binds to the mucosal addressin cell adhesion molecule (MAdCAM-1) expressed by blood vessels in the mucosa (Mowatt and Viney, 1997).

In the ileum of young horses, aggregates of lymphoid tissue have been shown to consist of three different morphological forms of follicle – follicle/dome structures, proprial follicles and lymphoglandular complexes (Lowden and Heath, 1995). The follicle/dome structure is the only follicle form described in the Peyer's patches in the small intestine of other animals; the lymphoglandular complexes have been described in the large intestine but not the small intestine in many other species (Lowden and Heath, 1995). However, the study was a structural not functional one and therefore it is not known what the impact (if any) is of the presence of these different follicle structures in the equine ileal lymphoid tissue on the equine mucosal immune response.

The method of lymphocyte isolation involved the inversion of a section of gut, followed by digestion from the mucosal surface inwards with EDTA to remove the

epithelium, and then collagenase to release lamina propria lymphocytes. To isolate cells from a Peyer's patch, the patch was dissected out of the gut section after removal of the epithelium, and then digested with collagenase. To compare cells isolated from Peyer's patches between horses, the chosen section of gut had to contain at least one Peyer's patch. However, unlike in the pig, equine Peyer's patches are not macroscopically visible from the serosal surface due to the muscular thickness of the equine small intestine. The presence of a Peyer's patch in the selected section of gut could only be confirmed when the gut had been inverted and filled with buffer. The procedure to this point was relatively time consuming - the muscular thickness of the gut caused problems both in tying knots that did not leak and in the actual inversion of the gut section, making repeated attempts at obtaining an appropriate section of gut impractical. To compare cells isolated from the lamina propria between horses, the chosen section of gut should not contain any Peyer's patches. The lamina propria contains a large number of B cells that are mainly terminally differentiated plasma cells. These cells would also be useful for investigating specific mucosal antibody responses. Lymphocytes were therefore isolated from either the Peyer's patches or lamina propria, depending on the presence or absence of Peyer's patches in the section of gut collected at the time. This would also enable comparison of the lymphocytes isolated from the two sites for their use in the ELISPOT assay.

The existence of a common mucosal immune system has been well documented (McDermott and Bienenstock, 1979; Weisz-Carrington et al, 1979; Czerkinsky et al, 1987; Forrest, 1992), with lymphocytes activated in the O-MALT also recirculating to distant mucosal tissues. However, it has also been observed that local exposure to an antigen can result in higher levels of specific IgA at the site of antigen-stimulation compared to more distant sites; higher levels of specific IgA within the intestine were

observed in the segment of intestine exposed to cholera toxin (Pierce and Cray, 1982) or poliovirus (Ogra and Karzon, 1969). BoNT/C and IgA specific for BoNT/C had been detected in the ileum of horses with grass sickness, (Chapters 3 and 6.1). However, it was not possible to use ileum sections for the isolation of lymphocytes as the muscle layers were too thick to allow inversion of the gut; jejunum, proximal to the ileum, was used instead. The ileum in the horse is relatively very short (approximately 1 metre in length) therefore it was thought that there would be little difference in the specificities of the cells isolated from the jejunum compared to those in the ileum.

Flow cytometric analysis of cells isolated from the equine GALT

Cells, collected at different stages in the lymphocyte isolation procedure, were analysed by flow cytometry to determine the percentage of lymphocytes isolated at the different stages and to determine the percentage of T and B cells within these populations. The ultimate aim of the isolation procedure was to isolate B cells that could be used in an ELISPOT assay. Therefore, it was essential to ensure that the procedure used was indeed isolating B lymphocytes.

Although the results obtained in this study were from individual experiments each using cells from a different horse, there were some common findings. Lymphocytes were detected in the EDTA wash medium, and these were presumably the intraepithelial lymphocytes (IELs). There was a higher percentage of T cells compared to B cells released at this stage. IELs are usually found between the enterocytes, separated from the gut lumen and lamina propria by the epithelial cell tight junctions and the basement membrane respectively. During the isolation procedure the EDTA causes the breakdown of the tight junctions between the epithelial cells. The last EDTA-wash medium contained between 19 and 38%

lymphocytes, with approximately 50% of the lymphocytes CD3+ and between 2 and 20% sIg+. However, this may reflect the distribution of cells closest to the basement membrane as it was the final wash; the cells were not pooled from all the EDTA-washes. In the pig, 27% of epithelial cells are CD2+ (Zhang, 1992, cited in Stokes et al, 1994); over 90% of the IELs express CD2 and of these 77% are CD8+ and 5% are CD4+ (Zhang,1992, cited in Stokes et al, 1994). In humans and rodents, the majority of IELs are also T cells, with approximately 85% CD8+ (Kraehenbuhl and Neutra, 1992). The IELs in the human are relatively enriched for the $\gamma\delta$ T cell receptor (Mowatt and Viney, 1997), but this is not the case in the pig (Stokes et al, 1994). The equine IELs were not characterised beyond CD3+ and sIg+ cells.

Collagenase breaks down the basement membrane thus releasing cells from the lamina propria or Peyer's patch. A greater percentage of lymphocytes were released by digestion of the lamina propria with collagenase, than by removal of the epithelium. The percentage of positively labelled T and B cells in the lymphocyte population also increased after collagenase digestion. The percentage of cells in the monocyte population was lower after collagenase digestion compared to cells released during removal of the epithelium. Collagenase digestion of the lamina propria released between 43-51% lymphocytes and 7-9% monocytes. Collagenase digestion of the Peyer's patch released 65% lymphocytes and 7% monocytes. 58-62% of the lamina propria lymphocytes were CD3+ T cells compared to 5-26% sIg+ B cells. Similar proportions of T and B cells were found in the Peyer's patch with 52% CD3+ and 18% sIg+. In mice, 40% of lamina propria lymphocytes were found to be sIgA+, and 25% were positive for T cell markers (Kraehenbuhl and Neutra, 1992).

As with the PBMCs, the CVS 36 antibody bound to a higher percentage of cells in the monocyte population than in the lymphocyte population. However, in terms of numbers, the antibody bound to a greater number of cells in the lymphocyte population. To ascertain whether the positively labelled cells in the monocyte population were due to non-specific binding or incorrect gating of the lymphocyte population on the bitmap, the monocytes were removed by adherence to plastic. In experiment four, removal of the monocytes left 5-6% of the remaining cells in the monocyte bitmap, therefore monocyte removal reduced the percentage of cells in this population by only a couple of percent. A decrease in the percentage of cells positively labelled with CVS 36 was also seen in the monocyte population, however the same decrease was observed in positively labelled cells in the lymphocyte population. The cells had been separated on Histopaque after removal of the monocyte and prior to flow cytometric analysis, therefore the differences in cell populations may be due to cells lost in the separation rather than due to removal of the monocytes. Removal of monocytes and separation on Histopaque increased the percentage of cells in the lymphocyte population by 12-26% percent for cells isolated from the Peyer's patch and lamina propria. The percentage of positively labelled T cells in the lymphocyte population also increased, but the percentage of B cells decreased. Even after removal of the monocytes in experiment four there were still more cells positively labelled with CVS 36 in the monocyte population than in the lymphocyte population. This suggests that either monocyte removal was not effective and this was non-specific binding or that indeed the lymphocyte population gate was incorrectly set. It may be that granular plasma cells were outside the gate that had been set for the lymphocytes. Whilst theoretically plasma cells do not express sIg, during differentiation it is likely that there is still some surface expression that could be bound by the CVS 36 antibody.

The cells were analysed after monocyte removal and prior to separation on Histopaque in experiment five. Unfortunately, it was not possible to compare cells before monocyte removal due to contamination of these cells. There were still 13% of cells in the monocyte region after monocyte removal, 68% of which were labelled with CVS 36 and 14% were labelled with anti-CD3. This would suggest that the lymphocyte gate was incorrect. However, analysis of the cells removed as 'monocytes' by adherence to plastic, demonstrated that only 7% of these cells were in the monocyte population as defined by the bitmap and 60% of the cells were in the lymphocyte population. This would suggest that the monocyte and lymphocyte populations are overlapping in the bitmap. However, the cells also showed very similar positive labelling with CVS 36 and anti-CD3 as the cells from which they had been removed, suggesting that monocyte removal had not been effective or that lymphocytes had also been removed. Subsequent to this experiment it was discovered that equine B cells can be separated from T cells by the preferential adherence of equine B cells to plastic (Crepaldi et al, 1986). This may account for B cells being present in the population of cells removed as 'monocyte', but it does not account for the high percentage of T cells in this group. It is possible that mixed cell aggregates may have formed.

It is unlikely that the lymphocyte population included non-lymphocytes as when anti-CD18 was used alongside the T and B cell markers, the percentage of positively labelled T and B cells was approximately equal to or greater than the number of leucocytes labelled with anti-CD18. If some lymphocytes were excluded from the lymphocyte population in the analysis then it is likely that the actual numbers of excluded cells were low as the percentage of cells in the monocyte region was not greater than 15% in any of the experiments. Therefore whilst there was a high percentage of cells binding CVS 36 and CD3 in the monocyte population, the actual

numbers of cells binding these antibodies in the monocyte population were very low; extension of the lymphocyte gate to include these cells did not significantly alter the percentage of positively labelled cells.

In order to determine whether there was non-specific binding by the monocytes, it would have been necessary to obtain a monocyte marker and look for double labelling of monocytes with CVS 36 and anti-CD3. However, there were no directly conjugated monocyte markers available, therefore preventing dual labelling analysis.

Although the primary aim of the flow cytometric analysis was to determine whether lymphocytes could be successfully isolated from equine GALT, some differences between horses were noted. In experiment one, the horse with CGS had a higher percentage of positively labelled T cells and lower percentage of B cells in the lymphocyte population than the healthy control. However, this is probably a reflection on variation in lymphocyte populations between horses, rather than a feature of equine grass sickness. There was no difference in the percentage of lymphocytes and monocytes in the PBMCs collected from these two horses. The horse in experiment two did not have grass sickness and had an equivalent percentage of T cells but lower B cells in the lymphocyte population compared to the horse with chronic grass sickness in experiment one. The horse with subacute grass sickness in experiment three, had a lower percentage of B cells in the lamina propria than the other horses investigated. More horses would have to be compared to determine whether there are changes in the T and B cell populations at either the systemic or mucosal level in horses with grass sickness.

Detection of immunoglobulin secreting equine B cells by ELISPOT

The ELISPOT assay, first described by Czerkinsky et al (1983) and Sedgwick and Holt (1983), is a modified ELISA that can detect antibody secreting cells in vitro; antigen-specific and total antibody secreting cells of a particular isotype can be determined by this assay. Initial ELISPOT assays were carried out using equine PBMCs as these were readily obtainable but the ultimate aim was to compare the specific- antibody secreting cells to *C. botulinum* type C and BoNT/C in the lamina propria or Peyer's patches of horses with and without grass sickness.

Total IgG-secreting cells (SC) were detected with PBMCs and cells isolated from the lamina propria and Peyer's patches. An ELISA had been developed for total IgG (Chapter 7.1.1) and the same reagents were used in the ELISPOT. However the capture antibody, anti-equine IgG, was titrated again to optimise the coating concentration; this might have differed between the two assays due to the different binding abilities of the cellulose wells in the ELISPOT plates, and plastic wells in the ELISA microtitre plates. Variable results were achieved with the different dilutions of capture antibody. Few ELISPOTs were detected when plates were coated with dilutions of capture antibody at 1 in 5,000 or higher; the 1 in 2,500 dilution appeared to be the highest dilution that gave detectable ELISPOTs.

The number of cells added to the wells appeared to be a crucial factor in influencing the amount of background staining. Initial assays with PBMCs, demonstrated that IgG secreting cells could be most accurately counted between 10^5 cells/well and 10^4 cells/well. Above 10^5 cells/well there was a lot of background, making the identification of true ELISPOTs difficult, and below 10^4 cells/well very few ELISPOTs were detected. This was also evident in the initial ELISPOT assay using cells isolated from the lamina propria and Peyer's patch, with too many spots to count

when $2x10^5$ cells/well or 10^5 cells/well added to wells coated with anti-horse IgG at dilutions of 1 in 2,500 or higher. For cells isolated from the lamina propria of another horse, total IgG secreting cells could be counted between 10^5 and 10^4 cells/well. The PBMCs from this horse had countable total IgG-secreting cells between 10^4 and $5x10^3$ cells/well. The variations in numbers of cells/well giving optimum numbers of ELISPOTs that are countable between horses, highlights the need to assay cells at two or three different dilutions.

Similar numbers of IgG-SC were detected in PBMCs and cells isolated from GALT. A mean of 39 (range 14-85) total IgG-SC/10^5 PBMCs could be detected by ELISPOT (capture antibody dilution 1 in 2,500; results from three horses). With cells isolated from GALT, the ELISPOT assay detected 37 total IgG-SC/10^5 lamina propria cells, reduced to 33 total IgG-SC/10^5 lamina propria cells if the cells were separated on Histopaque. In this horse, 5% of B cells isolated from the lamina propria (as determined by flow cytometric analysis) were detected as IgG-SC in the ELISPOT assay. Flow cytometric results were available for one of the assays using PBMCs: 2% of the total B cells secreted IgG in the ELISPOT assay. The detection of a lower percentage of IgG-SC in the circulating B cells compared to cells from the lamina propria, may reflect the large numbers of terminally differentiated plasma cells in the lamina propria.

The proportions of the different isotype-producing B cells in the equine lamina propria has not been published therefore it is not known whether the detection of 5% of lamina propria B cells as IgG secreting is an accurate reflection of the percentages of these cells. Certainly the ELISPOT assay will not detect all IgG-SC as not all will secrete IgG during the 4 h incubation period. In humans, IgA is the predominant immunoglobulin isotype in the mucosal immune system. However, the

studies on the immunoglobulin content of equine intestinal secretions showed that there was almost as much IgG present as IgA (Widders et al, 1984). If these immunoglobulin levels are reflected by the same proportions of antibody secreting cells in the lamina propria then this would suggest that the ELISPOT assay is detecting a low percentage of the IgG-secreting cells that are actually present. Other studies, using ELISPOT assays, express results as antibody secreting cells /number of cells added and do not compare them to real percentages of cells present, so it is difficult to compare the sensitivity of this assay with other published results. The ELISPOT assay to detect IgA secreting cells was not successful. High backgrounds were a problem making it impossible to detect the presence of true ELISPOTs.

Antigen-specific antibody secreting cells were detected, but in low numbers. One anti-*novyi* IgG-SC/10^5 Peyer's patches cells (0.08% of all B cells) and one anti-*novyi* IgG-SC/10^5 lamina propria cells (0.07% of all B cells) were detected in a horse with glomerulonephritis. Two anti-*C. botulinum* type C IgG-SC/10^5 lamina propria cells (0.03% of all B cells or 5% of total IgG-SC as detected by ELISPOT) were detected in a horse with suspected botulism. This horse had one anti-*novyi* IgG-SC/10^5 lamina propria cells, and one anti-BoNT/C/10^5 lamina propria cells. However, one anti-BSA IgG-SC/10^5 lamina propria cells was also detected in this horse. This may be evidence of non-specific binding to the nitrocellulose wells, or this horse may have had cross-reactive antibodies to BSA. Antibodies to BSA had not been detected in previous assays. The low numbers of specific ELISPOTs detected, demonstrates the need to add high numbers of cells to the wells when looking for specific antibody secreting cells. The numbers of cells that need to be added to detect a specific B cell are much greater than the numbers required for determining total numbers of IgG secreting cells. No specific antibody secreting cells were

detected in the PBMCs, reflecting low levels/no circulating B cells specific to these antigens.

In the one horse that had cells isolated from both the lamina propria and a Peyer's patch, there was little difference in total IgG-SC and specific IgG-SC between the cells from the two lymphoid sites. Separation of the GALT cells on Histopaque had little effect on the ELISPOT results. However, separation did result in a 2.5-5-fold decrease in total cell numbers. Flow cytometry demonstrated an increase in the lymphocyte population and decrease in the monocyte population after separation on Histopaque but there was also a decrease in the percentage of sIg+ cells. These results suggest that it is probably better not to separate the GALT cells on Histopaque prior to an ELISPOT assay.

Isolation of lymphocytes from the lamina propria and Peyer's patches followed by analysis of the antibody secreting cells by ELISPOT enabled demonstration of the presence of IgG-SC, as well as anti-*C. novyi* and anti-*C. botulinum* IgG-SC. However, on several occasions the ELISPOT assay was not possible due to contamination of cells during the isolation procedure. The horses used are not experimental animals but real clinical cases, often suffering from a GI-related disease. The conditions of the guts were therefore not always healthy, increasing the chances of microbial contamination of the cells. The isolation procedure is lengthy taking approximately 10 hours. The first incubation in the ELISPOT assay is for 4 h and often the isolated cells had to be kept in tissue culture flasks for 6-8h at 37°C in 5% CO_2, prior to starting the ELISPOT assay. This prolonged incubation also increased the chances of contamination. As stated previously the horses were not experimental animals and therefore their euthanasia could not be planned for.

To isolate the lymphocytes and carry out the ELISPOT in a reasonable time frame, the horse would have to be euthanased first thing in the morning, but this was usually not the case.

The ELISPOT has advantages over the ELISA, for example results are not affected by the absorption or elimination of antibody, or dilution factors of intestinal contents. However, the length of time required to isolate the cells from equine GALT followed by the ELISPOT assay, combined with the frequent contamination of cells, does not merit its use for the large scale investigation of mucosal immunity in equine clinical cases. It would however, be a useful technique for the future investigation of the mucosal immune responses, if experimental animals could be used. The appearance of antigen-specific IgA-SC in the peripheral blood, has been used by some investigators, as an indirect measure of the intestinal immune response to oral vaccination (Czerkinsky et al, 1987; Czerkinsky et al, 1991; Forrest, 1992). The appearance of these specific antibody secreting cells in the blood is transient and therefore this would not be a suitable method to use at this stage for the investigation of mucosal immunity to *C. botulinum* in equine grass sickness, as the time of exposure is not yet known.

6.2.4 Detection of antibodies in saponin extracted GALT

An alternative approach to investigating mucosal antibodies is the use of the detergent saponin to extract antibodies from mucosal GALT. Extraction of equine GALT tissue with saponin in the presence of protease inhibitors was effective at releasing antibodies detectable by both ELISA and RID. Saponin releases antibody from cells by solubilising the cell membranes. The IgG and IgA content of lamina propria tissue and Peyer's patch tissue from the same horse were very similar. This

correlates with the numbers of total IgG-SC isolated from the lamina propria and Peyer's patches as these were also very similar. However, there was a 50-fold difference in IgG levels between the two horses, which meant that samples should be assayed at more than one dilution for the calculation for total IgG to account for variation.

Extraction of GALT tissue with saponin resulted in a higher detectable level of IgG, compared to protease treated ileum contents and faeces. However this was not the case with the detection of total IgA as one horse had no detectable IgA in the GALT extracts. IgA in GALT extracts, from the second horse investigated, could be quantified by RID, whereas the IgA in a sample of ileum contents was below the threshold concentration for detection by RID.

The extraction method was less time consuming than the ELISPOT assay, and the ELISA results were easier to interpret compared to the enumeration of ELISPOTs. The extraction method also has an advantage over the detection of IgA in ileum contents and faeces as the results should not be affected by antibody fluctuations due to absorption and elimination of antibody. Degradation of extracted antibody by proteases should be less compared to protease degradation of antibody in intestinal contents or faeces, as the extracted antibody will not have been in contact with the gut contents.

A possible criticism of this method of investigation of the mucosal immune response is the potential contamination of extracted antibody with systemic antibody derived from blood in the tissues. Although this factor was not investigated here, previous studies by other investigators have shown this to be negligible. In humans, IgA levels in extracted biopsies correlated with IgA-SC from mucosal biopsies but not

with IgA levels in plasma (Bergquist et al, 2000). Studies in mice showed that there was less than 2% systemic antibodies in mucosal extracts (Bergquist et al, 1995; Johansson et al, 1998).

Although the levels of IgA could not be calculated by ELISA, the value obtained by RID was almost the same as the IgG levels. A previous study had shown that there was slightly higher IgA in intestinal contents (0.08mg/ml) compared to IgG (0.07mg/ml) determined by RID (Widders et al, 1984). In this study, in horse two, the IgG level was 2.1mg/ml in the extract from the lamina propria and 2.8mg/ml in the extract from the Peyer's patches, compared to an IgA level of 1.5mg/ml both in extracts from the lamina propria and Peyer's patches. These differences may be a reflection of differences in the two techniques used. The anti-equine IgG used in the ELISA for total IgG was raised against the light chain as well as the heavy chain of IgG and therefore may detect other equine isotypes (as discussed in Chapter 7).

Solubilisation of GALT with saponin and subsequent analysis of released antibodies by ELISA may prove to be a more efficient method for the investigation of specific mucosal antibody responses in clinical cases of grass sickness. However, the detection of IgA in the faeces of horses has a major advantage over both the ELISPOT and saponin extraction techniques, in that it can compare antibody levels in the GI tract pre-mortem, an important factor to consider in the investigation of non-fatal clinical cases.

Chapter Seven

Detection of IgG and IgA in colostrum and milk to *C. botulinum* type C surface antigens and BoNT/C

7.1 Results

7.1.1 Optimisation of assay for total IgG ELISA

Capture antibody

Two rabbit anti-equine IgG (H+L) antisera (Nordic and ICN) were compared for use as a capture antibody for an ELISA to detect total equine IgG. ELISA plates were coated with each antisera diluted fivefold from 1 in 20 to 1 in 12500. An equine serum sample was used to compare which capture antibody gave optimal binding. The serum sample was serially diluted fivefold, between 1 in 4 and 1 in 500, and titrated against the capture antibody. Approximately three-fold higher OD values were observed when the ICN affinity-purified rabbit anti-equine IgG (H+L) antiserum was used as the capture antibody, compared to the Nordic antiserum (Fig. 7.1). Therefore, it was decided to use the ICN antiserum as the capture antibody and experiments were carried out to optimise this assay. Equine IgG was titrated against a twofold dilution series of the ICN anti-equine IgG (H+L), from 1 in 2500 to 1 in 200000. The 1 in 2500 dilution of capture antibody gave the highest OD readings at 0.1 and 0.05 μg/ml IgG (Fig. 7.2). It was decided to use the ICN anti-equine IgG antiserum at a dilution of 1 in 2500 (protein concentration of 0.8μg/ml) for the coating of further ELISA plates (Fig. 7.2).

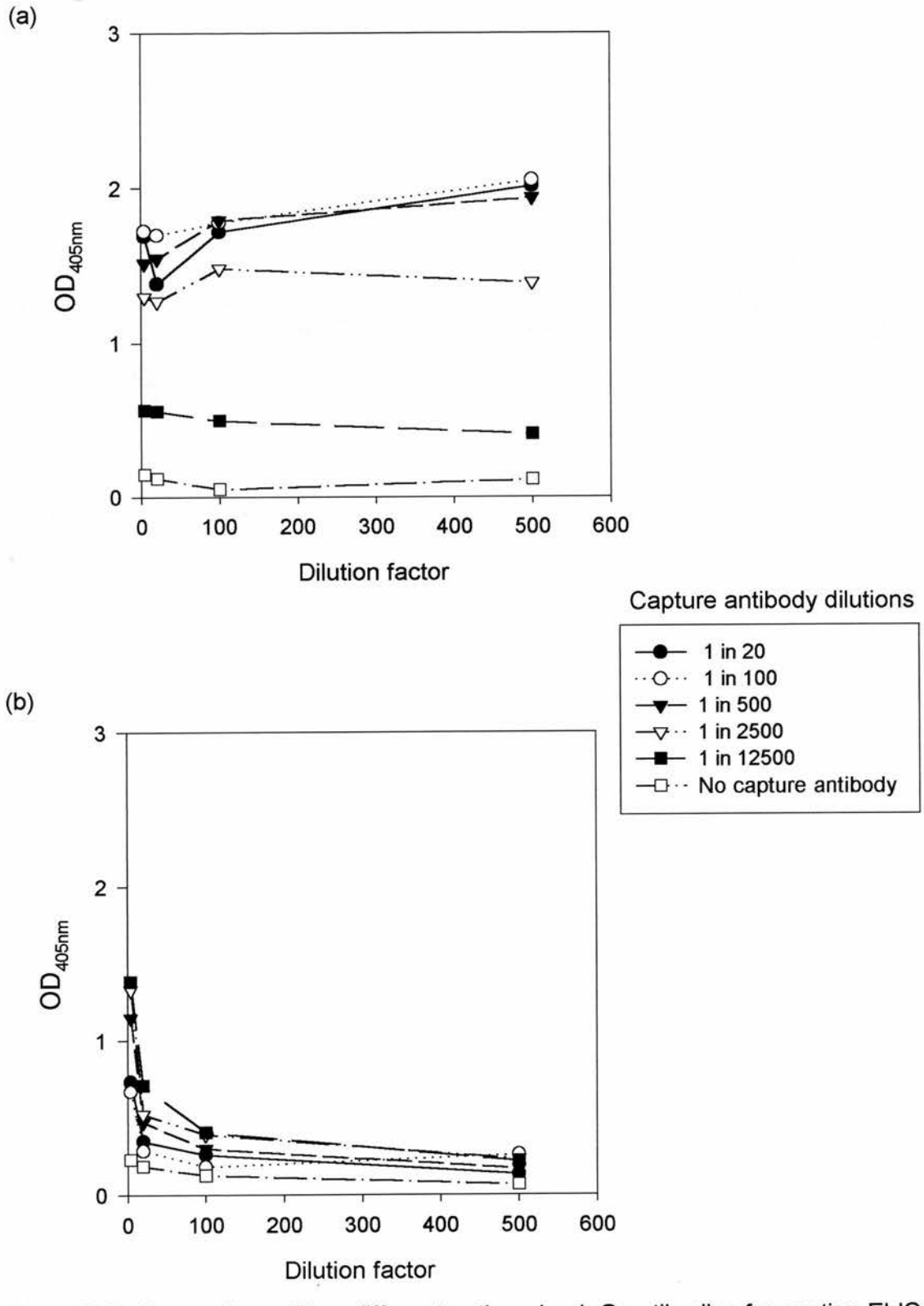


Figure 7.1: Comparison of two different anti-equine IgG antibodies for coating ELISA plates for the detection of total IgG in a serum sample.
(a) Plates were coated with five dilutions of anti-equine IgG (H+L) antiserum (ICN) and (b) anti-equine IgG (H+L) antiserum (Nordic). Serum sample was diluted fivefold between 1 in 4 and 1 in 500.

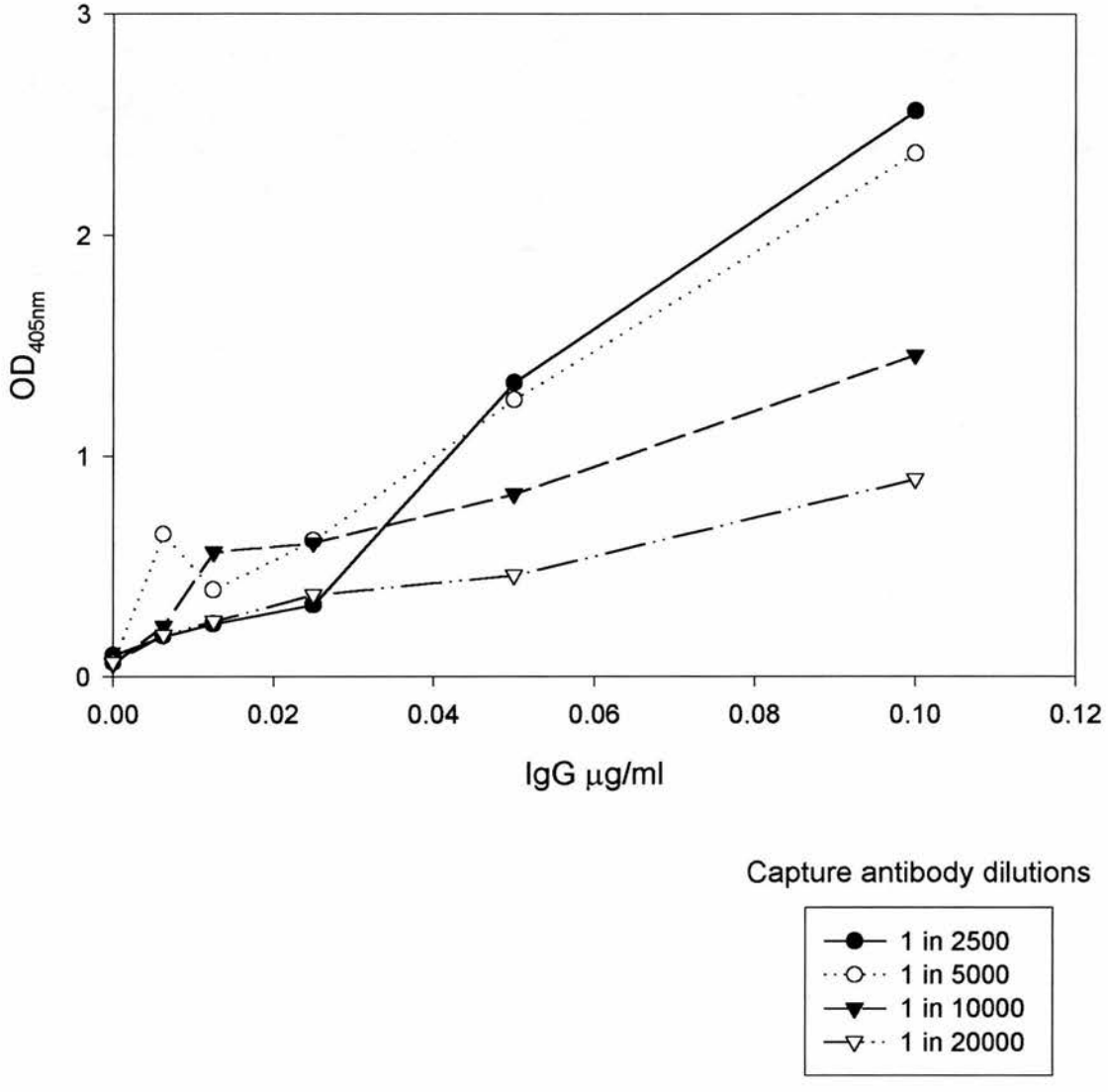


Figure 7.2: Equine IgG titrated against different coating dilutions of anti-equine IgG antiserum (ICN). Capture antiserum was diluted twofold between 1 in 2,500 and 1 in 20,000.

IgG standard curve

In order to calculate the concentration of IgG in each sample, it was necessary to have a positive control of a known IgG concentration. Equine IgG was obtained from an equine serum sample using a protein G column (MAb Trap GII kit, Pharmacia Biotech). The eluted IgG fraction had a protein concentration of 2.94mg/ml and this was taken to represent the IgG concentration. In order to determine the dilution range that would give a linear relationship, the equine IgG standard was initially diluted tenfold from 1000μg/ml to 0.001μg/ml, then fivefold from 1μg/ml to 0.00032μg/ml, and finally twofold from 0.2μg/ml to 0.0008μg/ml. It was established that dilution of the equine IgG sample twofold from 0.1μg/ml to 0.003μg/ml produced a standard curve with a linear relationship (Fig. 7.3). The equation of the line could then be used to calculate the IgG concentration of the samples assayed on the ELISA plate.

Dilution of colostrum and milk samples

Preliminary assays were carried out to determine the optimum dilutions for assaying colostrum and milk samples for total IgG. The OD values for these samples had to fall within the linear part of a titration curve, be above the background level of the ELISA (i.e. greater than the mean of the blank control + 3 x S.D.) and within the range of the standard curve for total IgG.

Initial fivefold dilutions of two colostrum samples, between 1 in 100 and 1 in 312500, demonstrated that they would have to be titrated further. The same was observed when three colostrum samples were diluted twofold from 1 in $5x10^4$ to 1 in $1.6x10^6$. Finally, a decrease in OD was observed when three colostrum samples were diluted fivefold from 1 in $1x10^6$ to 1 in $3.125x10^9$ (Fig. 7.4). Twelve colostrum samples were

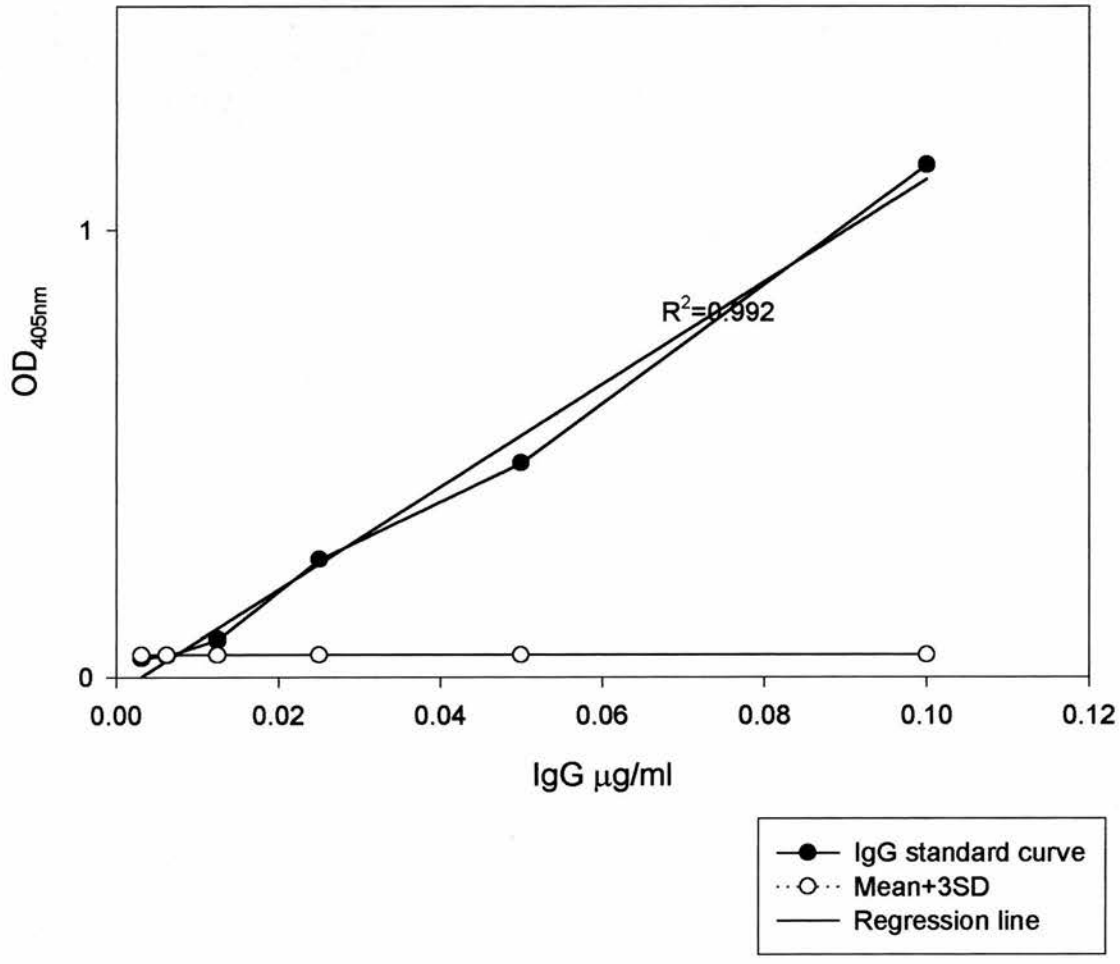


Figure 7.3: ELISA for total IgG. Twofold dilutions of equine IgG standard from 0.1µg/ml to 0.003µg/ml

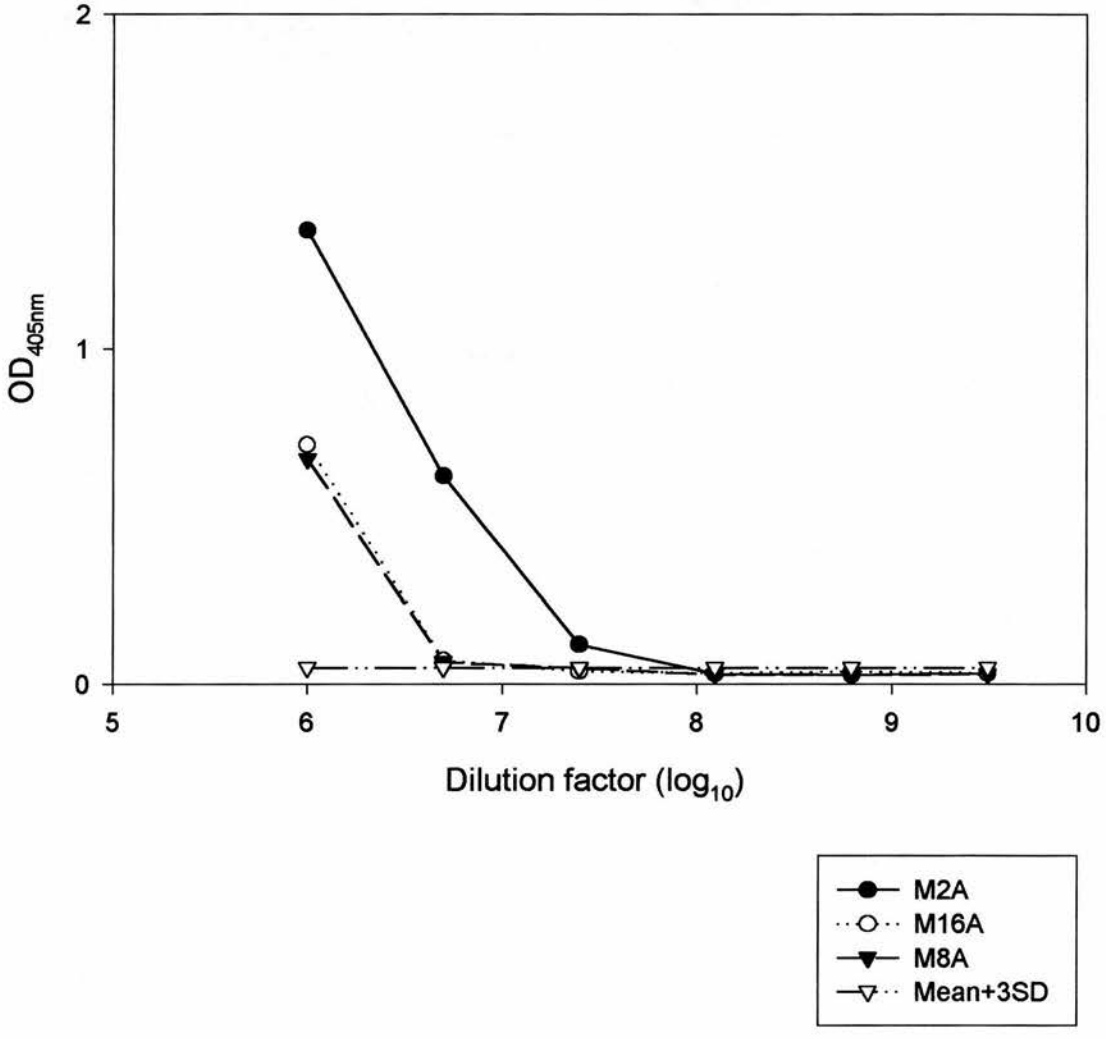


Figure 7.4: Total IgG in three colostrum samples diluted from 1 in 1,000,000 to 1 in 3,125,000,000

diluted tenfold from 1 in 100 to 1 in 10^7 to determine the optimum dilution for screening all the colostrum samples for total IgG (Fig. 7.5a and b). However, due to the variability observed in the IgG content of these samples it was decided that it would be more accurate to assay each sample at four dilutions. Colostrum samples were therefore diluted fivefold between 1 in 10^5 to 1 in 1.25×10^7, a dilution curve plotted, and the most appropriate dilution for each sample chosen to calculate the total amount of IgG in each sample (Fig. 7.6).

Three milk samples collected at two weeks, four weeks and eight weeks post-parturition were assayed at fivefold dilutions from 1 in 20 to 1 in 6.25×10^4. A prozone effect was observed up to the 1 in 500 dilution, but there was little variation in the IgG content between two and eight weeks (Fig. 7.7a). Subsequently three milk samples collected at two weeks post-parturition were diluted fivefold from 1 in 400 to 1 in 1250000 (Fig. 7.7b).

Due to the variation observed in IgG levels in colostrum and milk samples between mares (Fig. 7.5 and 7.7b), it was decided to screen all milk samples at four dilutions. Milk samples were therefore diluted fivefold between 1 in 1000 and 1 in 125,000 and a dilution curve was plotted for each sample (Fig. 7.8). From this dilution curve, the most appropriate dilution was chosen to calculate the amount of IgG present in the sample.

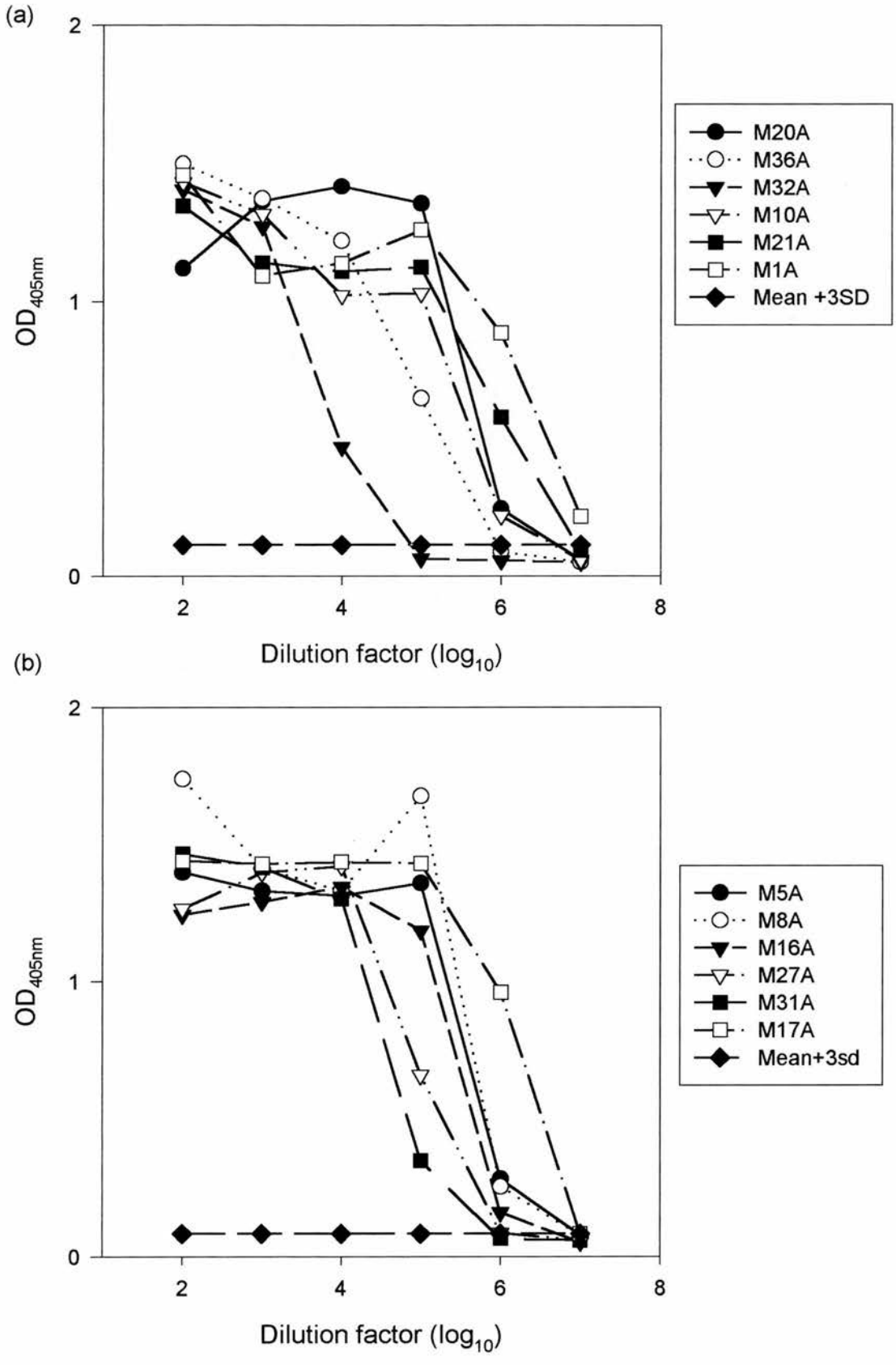


Figure 7.5(a) and (b) - Total IgG in twelve colostrum samples diluted tenfold from 1 in 100 to 1 in 10,000,000

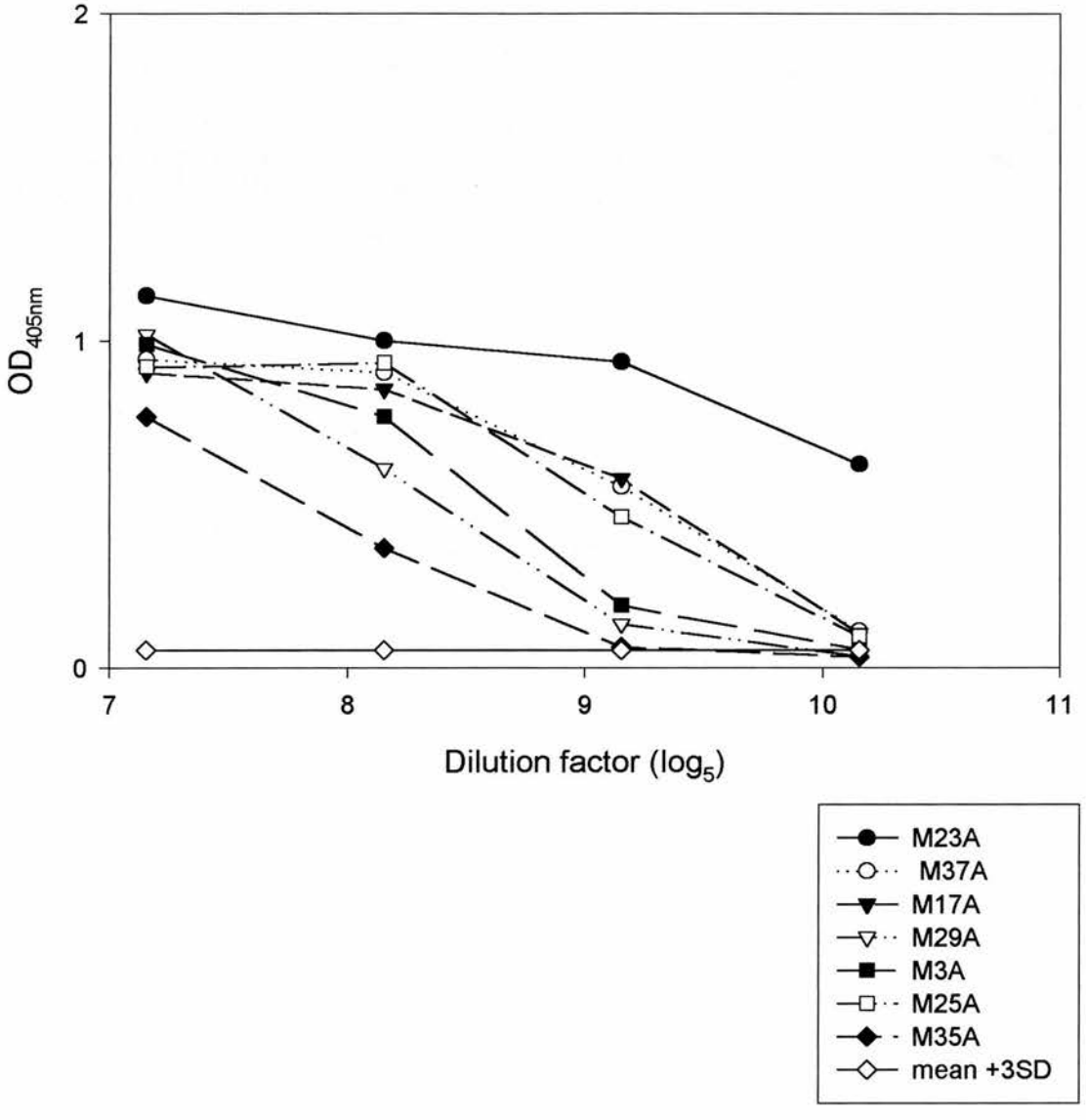


Figure 7.6: Total IgG in seven colostrum samples. Fivefold dilutions of colostrum from 1 in 100,000 to 1 in 12,500,000

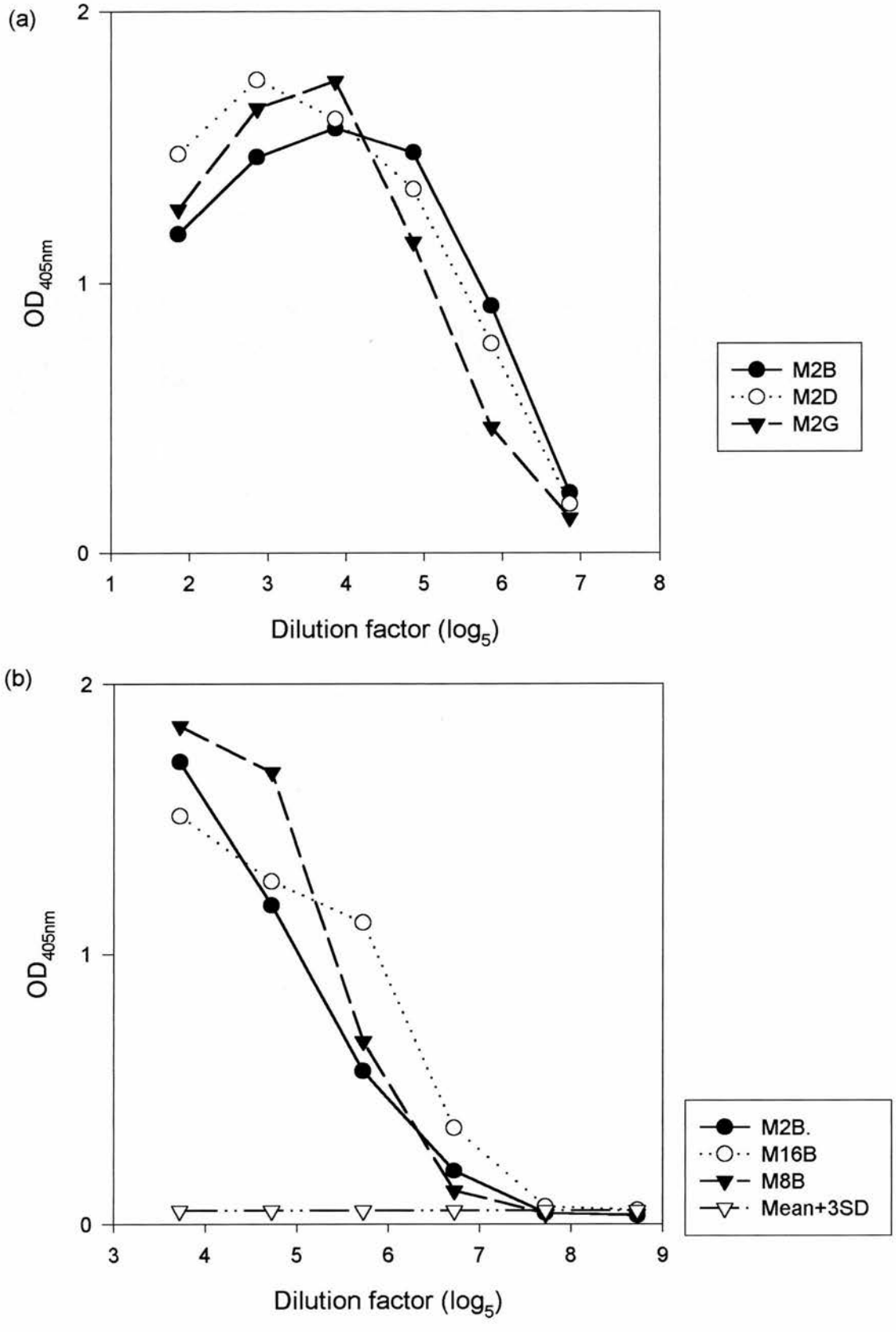


Figure 7.7: Total IgG in (a) three milk samples collected at 2, 4 and 8 weeks post-parturition, diluted fivefold from 1 in 20 to 1 in 62,500 and in (b) three milk samples, all collected at 2 weeks, diluted from 1 in 400 to 1 in 1,250,000.

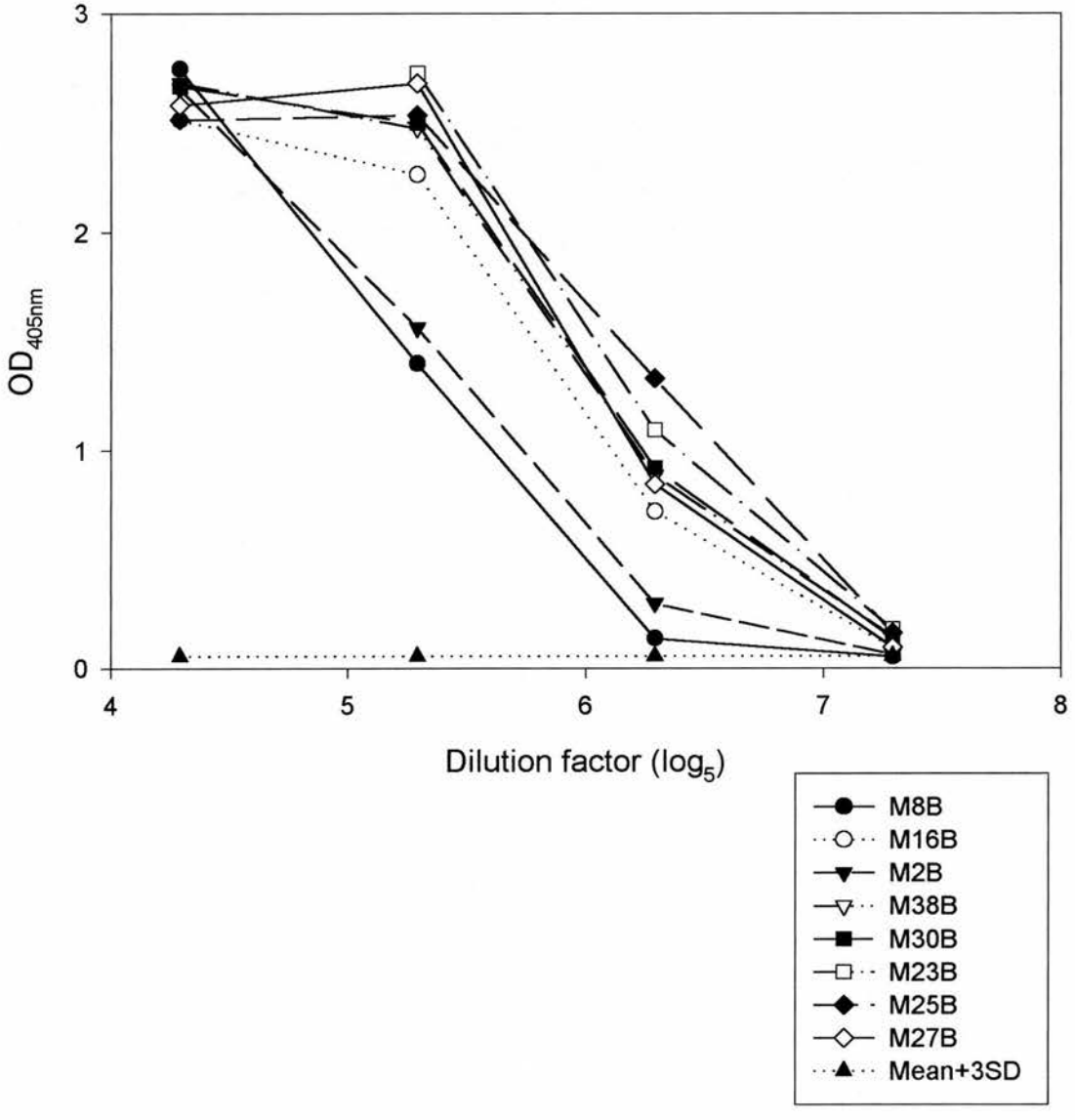


Figure 7.8: Total IgG in eight milk samples collected at 2 weeks, diluted fivefold from 1 in 1,000 to 1 in 125,000

7.1.2 Optimisation of ELISA for total IgA in colostrum and milk samples

Capture antibody and primary detecting antibody

Various combinations of anti-equine IgA antibodies were investigated for use as the capture (coating) antibody and the primary detecting antibody for an ELISA to determine total IgA (there was no directly conjugated anti-equine IgA antibody available). These antibodies were titrated to establish the optimum dilutions for the ELISA. A colostrum sample was used as the positive control to optimise the assay. Table 7.1 illustrates the various combinations of reagents and dilutions used.

Initially, plates were coated with anti-horse IgA (Fc) rabbit antiserum (Nordic) or anti-horse IgA mouse monoclonal (BVS), with the ISL mouse monoclonal as the primary detecting antibody (combinations 1 and 2 in Table 7.1). High backgrounds were observed when the BVS monoclonal was the capture antibody, with the sample OD values only slightly higher than background. This was probably due to the anti-mouse conjugate binding to the mouse monoclonal capture antibody: using a capture antibody and primary detecting antibody of the same host species is not a suitable combination for the ELISA. When the rabbit antiserum was used as the capture antibody (combination 2) the backgrounds were lower, but the sample OD values were also very low.

Different combinations of capture antibody and primary detecting antibody were therefore investigated. Plates were coated with the anti-horse IgA (Fc) rabbit antiserum (Nordic), with the BVS mouse monoclonal as the primary detecting antibody (combination 3). However, this combination still gave very low OD levels. When these antibodies were reversed, with the BVS monoclonal used as the

Table 7.1: Optimisation of ELISA for total IgA. The table describes the different combinations of capture antibodies and primary detecting antibodies used to optimise the assay.

Combination	Capture antibody	Capture antibody dilutions	Colostrum sample	Primary detecting antibody	Primary detecting antibody dilutions	Conjugate and dilution
1	Anti-horse IgA (Fc) rabbit antiserum (Nordic)	Fivefold: 1 in 20 to 1 in 12500 Twofold: 1 in 25 to 1 in 80000	Fivefold: 1 in 4 to 1 in 500 1 in 4 to 1 in 2500	Anti-horse IgA mouse m/c[1] (ISL)	1 in 200	Anti-mouse IgG AP[2] 1 in 5000
2	Anti-horse IgA mouse m/c (BVS)	Fivefold: 1 in 20 to 1 in 12500 Twofold: 1 in 200 to 1 in 25600	Fivefold: 1 in 4 to 1 in 500 1 in 4 to 1 in 2500	Anti-horse IgA mouse m/c (ISL)	1 in 200	Anti-mouse IgG AP 1 in 5000
3	Anti-horse IgA (Fc) rabbit antiserum (Nordic)	Twofold: 1 in 200 to 1 in 25600	Fivefold: 1 in 20 to 1 in 500	Anti-horse IgA mouse m/c (BVS)	Twofold: 1 in 100 to 1 in 400	Anti-mouse IgG AP 1 in 5000
4	Anti-horse IgA mouse m/c (BVS)	Twofold: 1 in 200 to 1 in 25600	Fivefold: 1 in 20 to 1 in 500	Anti-horse IgA (Fc) rabbit antiserum (Nordic)	Fivefold: 1 in 100 to 1 in 2500	Anti-rabbit IgG AP 1 in 5000
5	Anti-horse IgA mouse monoclonal (BVS)	Fivefold: 1 in 200 to 1 in 15,625,000	1 in 100 and 1 in 1000	Anti-horse IgA (Fc) rabbit antiserum (Nordic)	1 in 1000	Anti-rabbit IgG AP 1 in 5000 and 1 in 10000
6	Anti-horse IgA mouse m/c (BVS)	1 in 1000	Tenfold: 1 in 100 to 1 in 1,000,000	Anti-horse IgA (Fc) rabbit antiserum (Nordic)	Twofold: 1 in 200 to 1 in 6400	Anti-rabbit IgG AP 1 in 5000

[1]m/c=monoclonal [2]AP=alkaline phosphatase

capture antibody and the Nordic rabbit antiserum used as the primary detecting antibody (combination 4), the OD readings for IgA in the colostrum sample were significantly higher. Therefore, further work concentrated on optimising the ELISA using the anti-horse IgA mouse monoclonal (BVS) as the capture antibody and the anti-horse IgA (Fc) rabbit antiserum (Nordic) as the primary detecting antibody (combinations 5 and 6). From these experiments, it was established that the capture antibody should be used at a dilution of 1 in 1000 (Fig. 7.9a), the primary detecting antibody at 1 in 400 (Fig. 7.9c) and the conjugate at 1 in 5000 (Fig. 7.9a & b). Using the capture antibody at a dilution of 1 in 1000 gave high OD levels and was reasonably economical. When the conjugate was used at 1 in 5000 a threefold higher OD was obtained for the sample than when the conjugate was used at 1 in 10000. The primary detecting antibody dilution of 1 in 400 was chosen because this dilution gave the highest OD without high background OD reading.

IgA standard

In order to calculate the amount of total IgA in each sample, a positive control of known IgA concentration was required. The amount of IgA in a selected colostrum sample (M16A) was found to be 6mg/ml using a RID kit (LLA RID kit, VMRD, Inc.). This colostrum sample was used as a positive control and titrated in the ELISA for total IgA. The sample was diluted tenfold between 100μg/ml and 0.001μg/ml and twofold between 10μg/ml to 0.3125μg/ml, in order to establish the appropriate dilution range to achieve a standard curve. A logarithmic relationship (log_2) was observed between 10μg/ml and 0.15μg/ml IgA. It was therefore decided to dilute the colostrum sample twofold, between 10μg/ml and 0.15μg/ml IgA to produce a standard curve on each ELISA plate (Fig. 7.10).

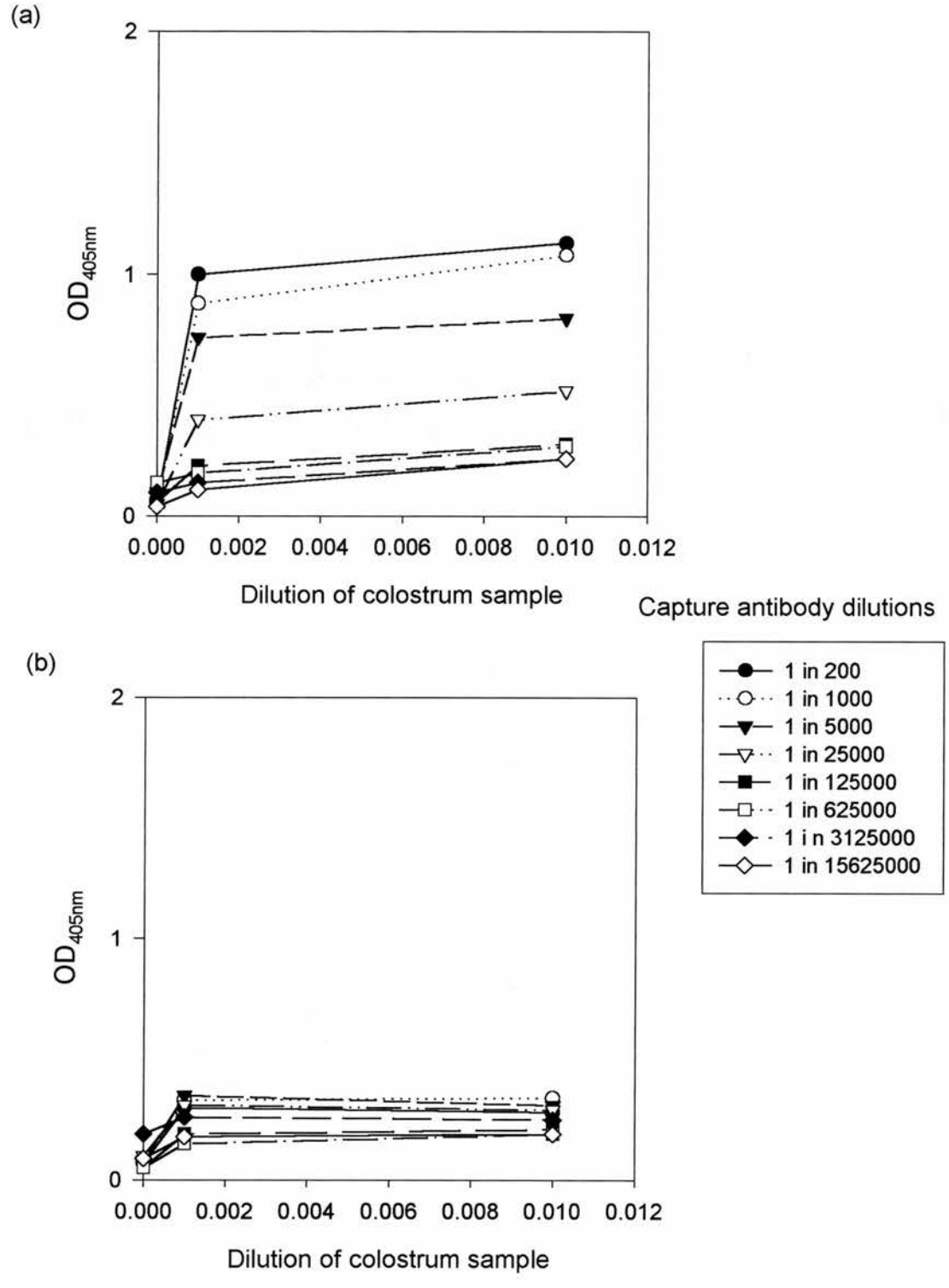


Figure 7.9: ELISA for total IgA. Fivefold dilutions of capture antibody, monoclonal anti-equine IgA (BVS) from 1 in 200 to 1 in 15,625,000 with (a) the conjugate diluted 1 in 5,000 and (b) 1 in 10,000. The colostrum sample was used at dilutions of 1 in 100 and 1 in 1000. The graphs also show the OD when no colostrum sample was added.

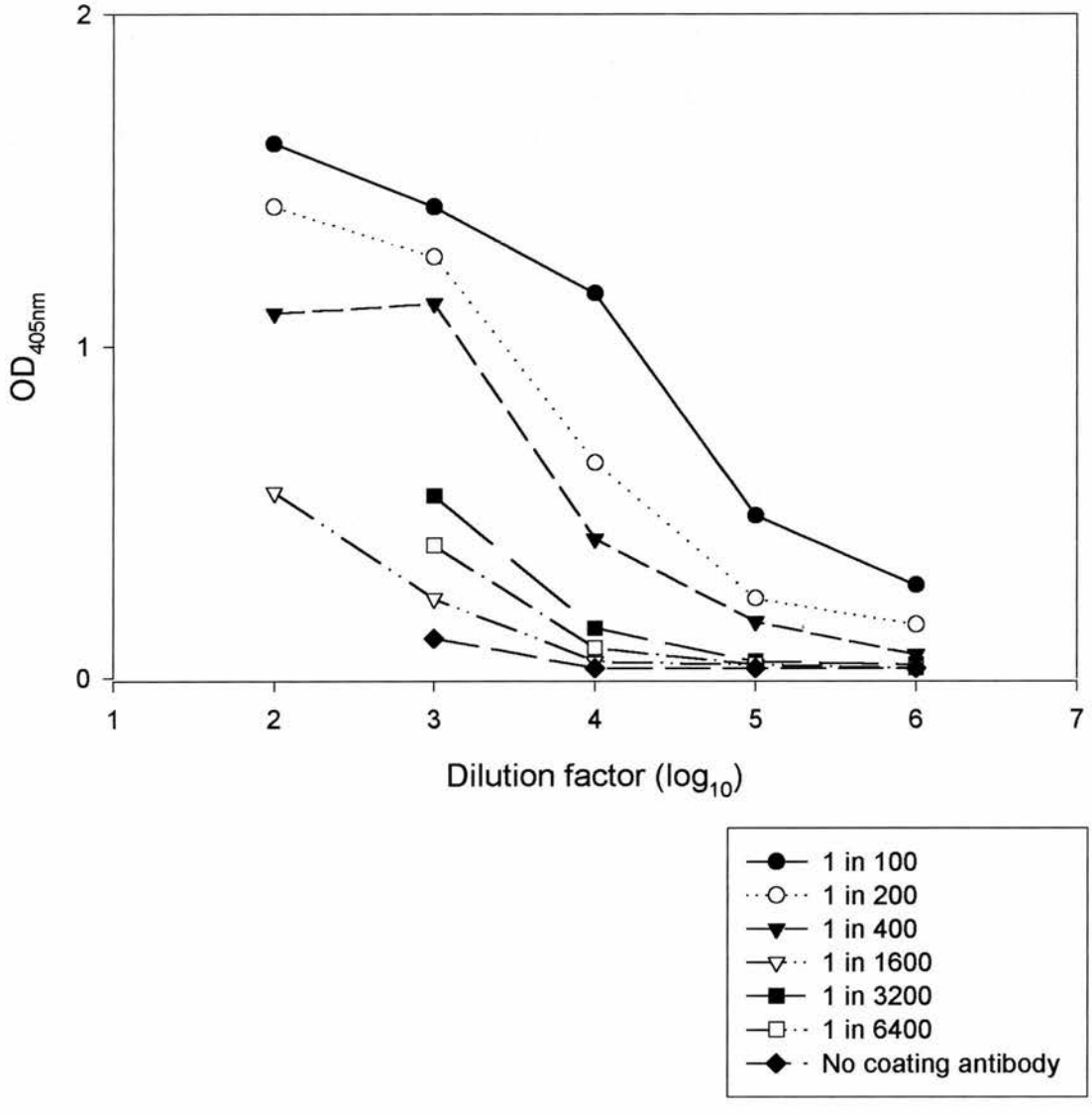


Figure 7.9(c): ELISA for total IgA. Twofold dilutions of rabbit anti-equine IgA antiserum as primary detecting antibody, from 1 in 100 to 1 in 6,400 with BVS capture antibody at 1 in 1,000 and conjugate diluted 1 in 5,000. The colostrum sample was diluted tenfold between 1 in 100 and 1 in 1,000,000.

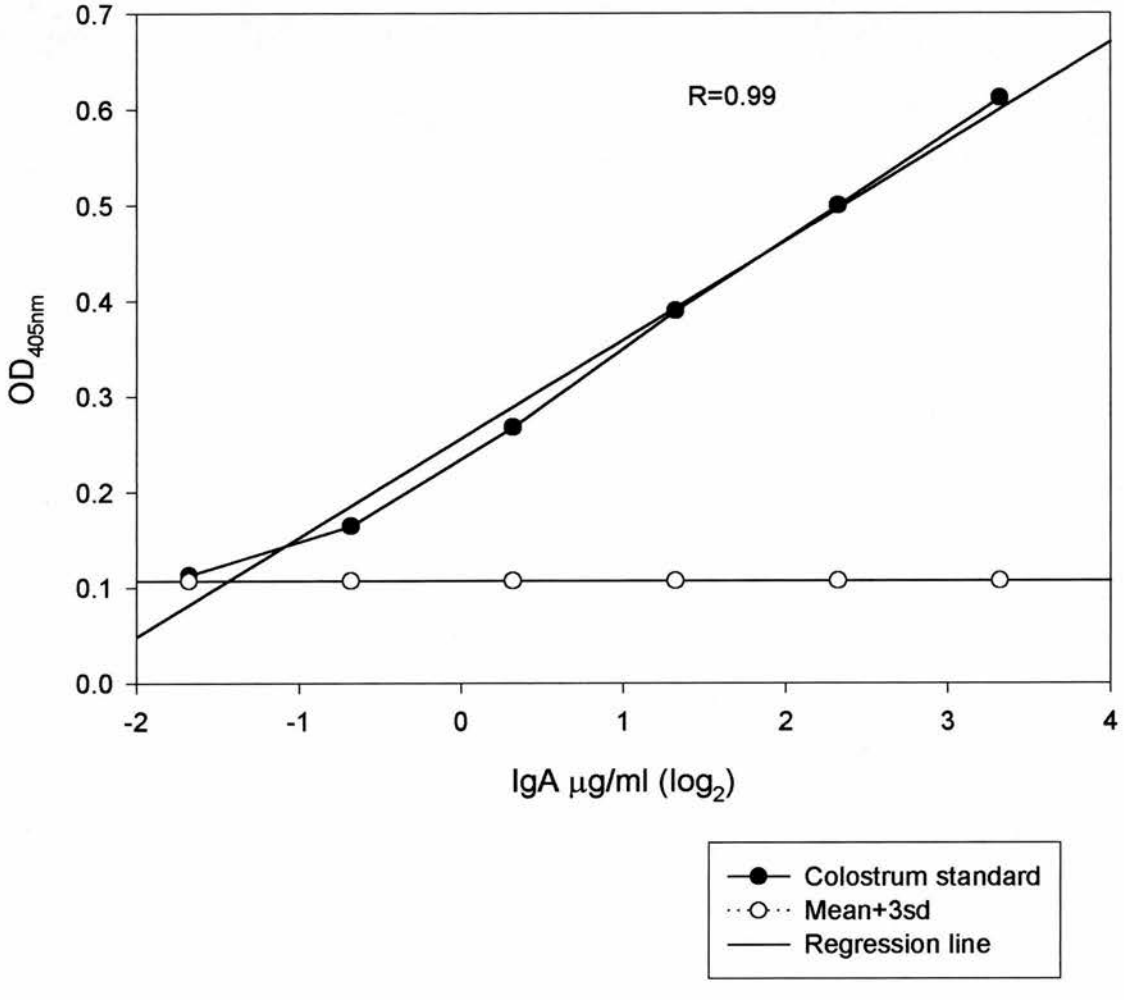


Figure 7.10: Total IgA in colostrum standard. Colostrum standard diluted twofold between 10 and 0.3125 µg/ml.

Dilution of colostrum and milk samples for determination of total IgA

Twelve colostrum samples were diluted between 1 in 500 and 1 in 16000 to establish a suitable dilution for screening samples (Fig. 7.11a). However, only two colostrum samples were significantly above background in this dilution range and these were further titrated out fivefold from 1 in 500 to 1 in 1562500 (Fig. 7.11b). Eight other colostrum samples were diluted fivefold from 1 in 10 to 1 in 1250 (Fig. 7.11c). All samples had positive OD values at 1 in 10 dilution. These results showed that there was considerable variation in IgA levels between the colostrum samples. Therefore, all the colostrum samples were diluted tenfold and assayed at 4 dilutions between 1 in 10 and 1 in 10000 (Fig. 7.11d). A dilution curve was plotted and the most appropriate dilution was chosen for the calculation of total IgA in each sample.

All milk samples were diluted fivefold and assayed at four dilutions from 1 in 5 to 1 in 625. A dilution curve for each sample was plotted and the most appropriate dilution chosen for calculation of total IgA (Fig. 7.12).

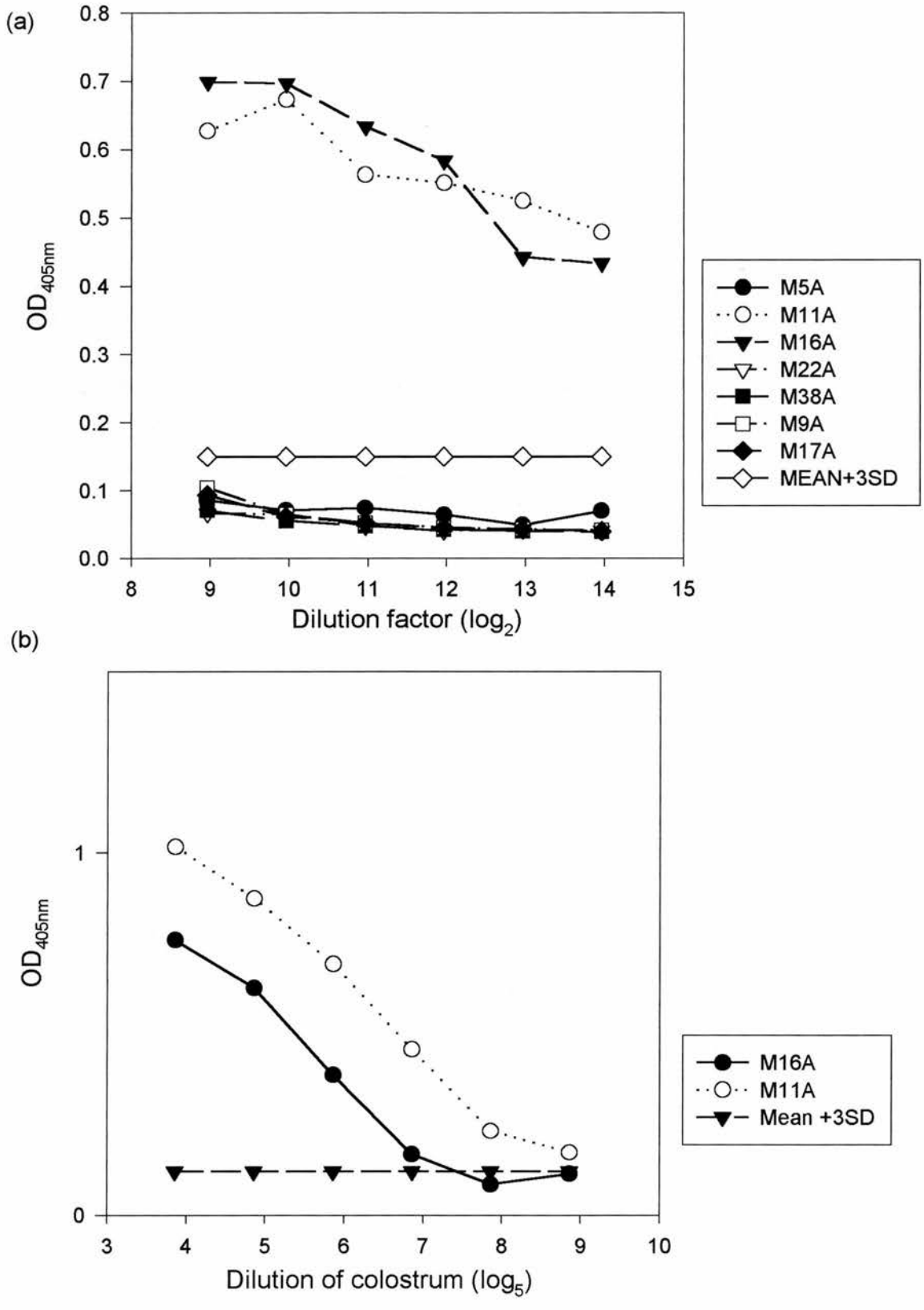
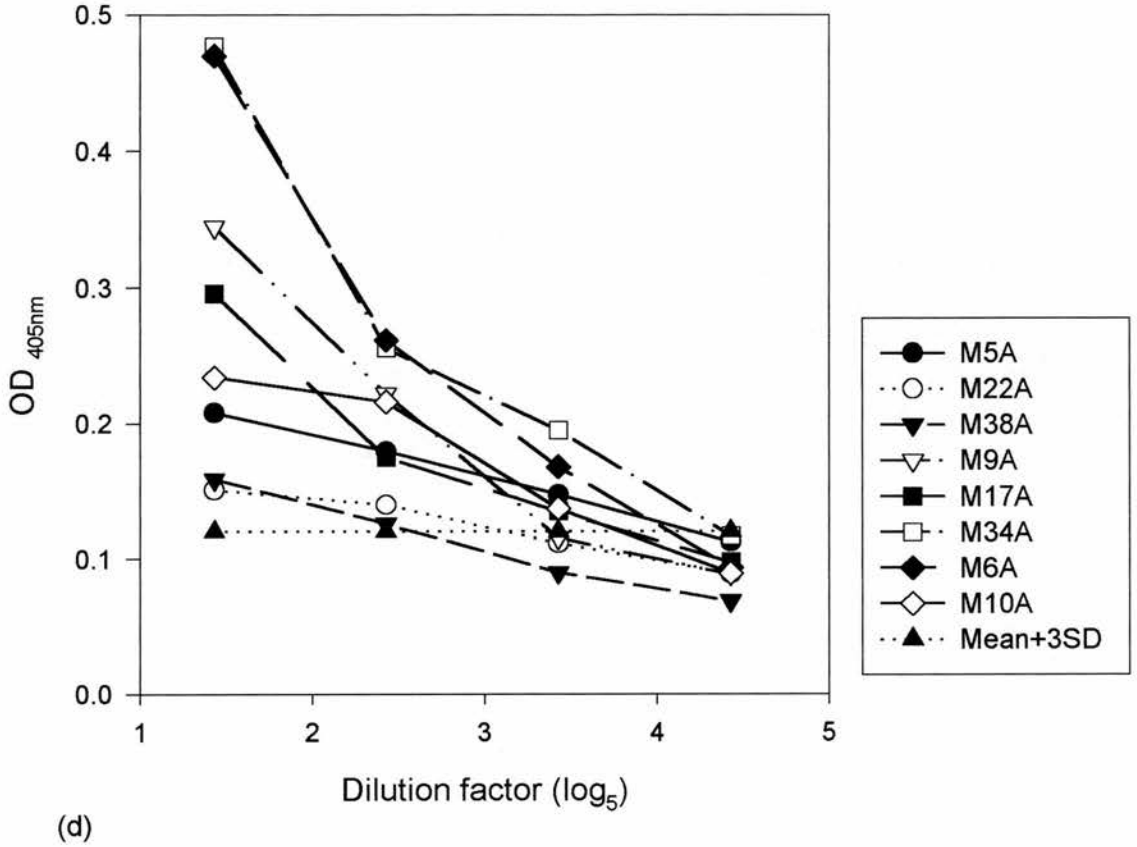
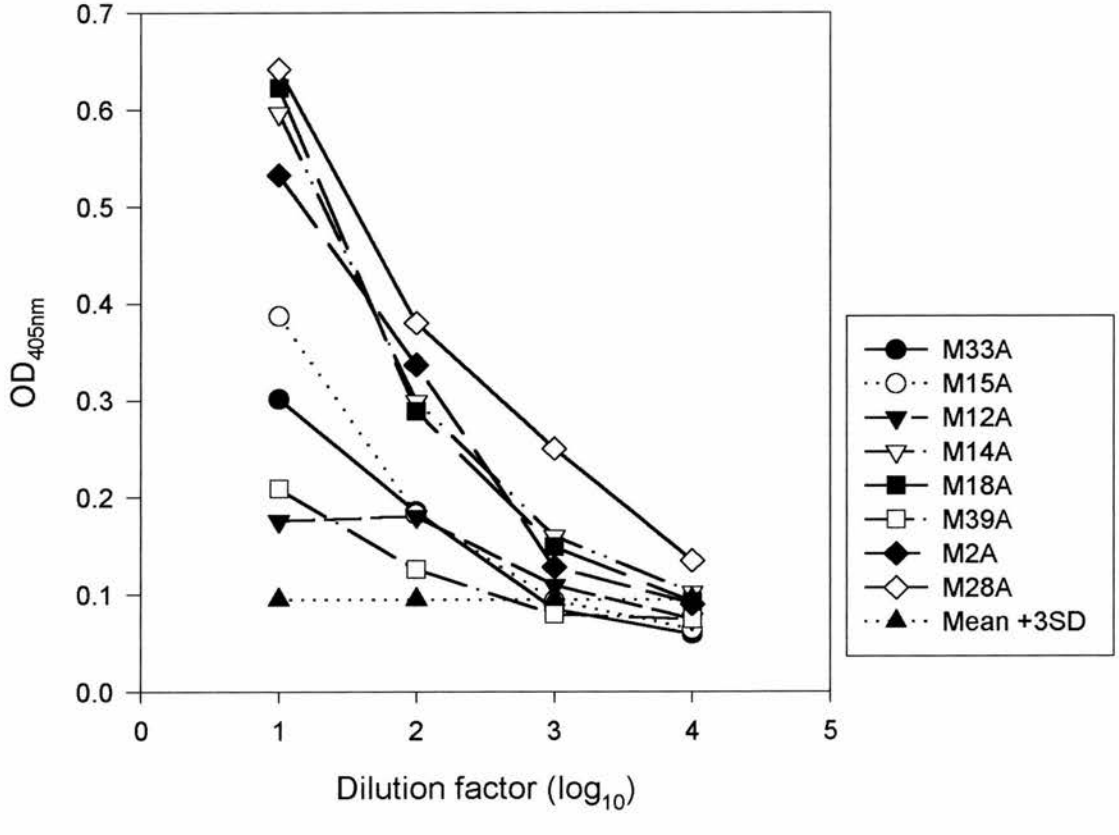


Figure 7.11: Total IgA in colostrum samples (a) colostrum samples diluted twofold between 1 in 500 and 1 in 16,000, (b) colostrum samples diluted fivefold between 1 in 500 and 1 in 1,562,500, (c) colostrum samples diluted fivefold between 1 in 10 and 1 in 1,250 and (d) colostrum samples diluted tenfold between 1 in 10 to 1 in 10,000.

(c)


0.5
0.4
0.3
0.2
0.1
0.0
OD 405nm
1
2
3
4
5
Dilution factor (log5)
M5A
M22A
M38A
M9A
M17A
M34A
M6A
M10A
Mean+3SD

(d)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD405nm
0
1
2
3
4
5
Dilution factor (log10)
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M15A
M12A
M14A
M18A
M39A
M2A
M28A
Mean +3SD

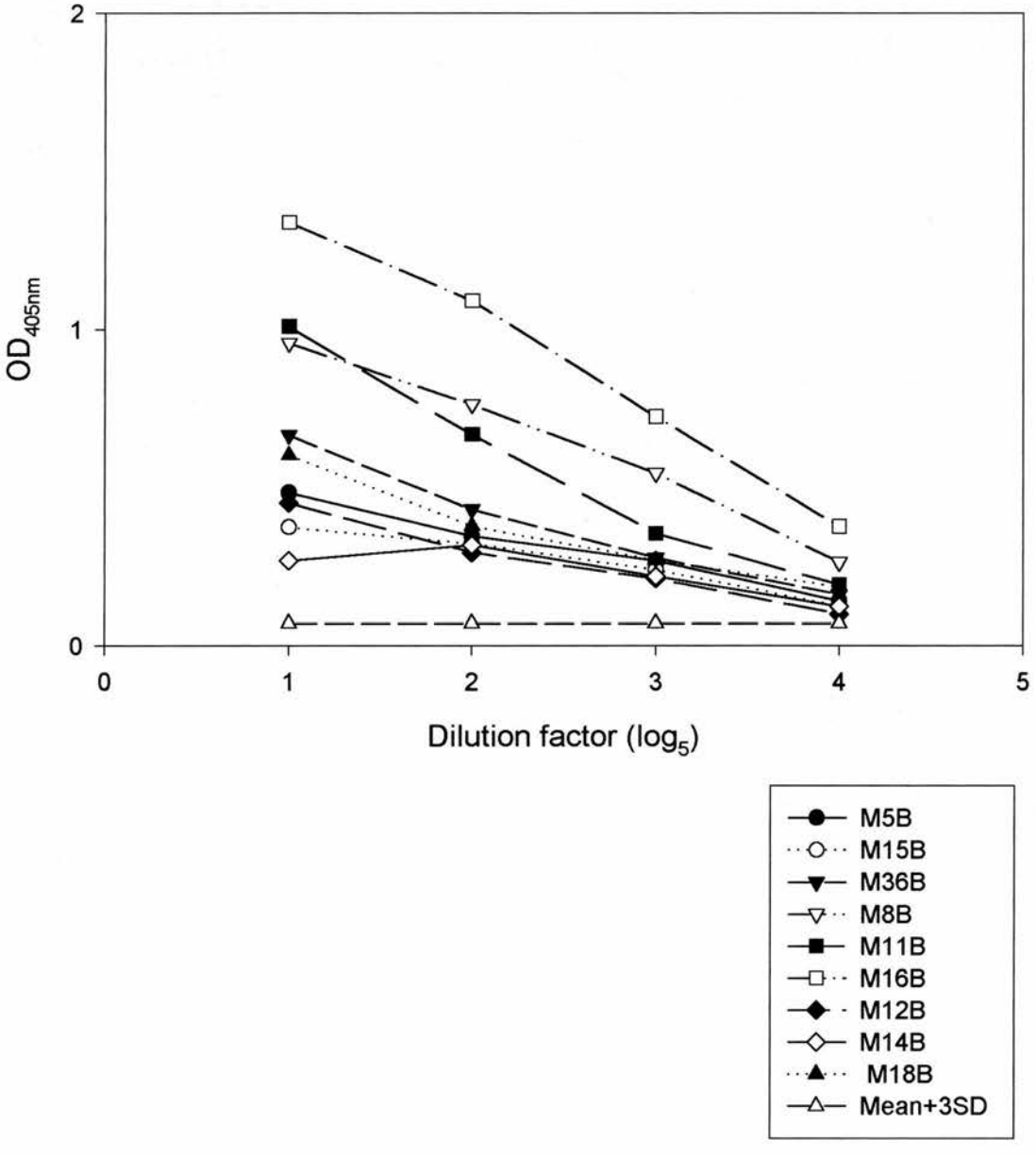


Figure 7.12: Total IgA in nine milk samples. Fivefold dilutions of milk samples from 1 in 25 to 1 in 625.

7.1.3 Optimisation of ELISAs for detection of IgG and IgA surface antigens and BoNT/C

Dilution of colostrum and milk samples for IgG screening assays

Preliminary assays were carried out to establish the optimal dilutions of colostrum and milk samples for a screening assay to detect IgG to surface antigens and BoNT/C. Initially, eight colostrum samples and 14 milk samples (collected at intervals over the suckling period) were diluted fivefold between 1 in 4 to 1 in 100, for both the ELISA for IgG to surface antigens (Fig. 7.13) and to BoNT/C (Fig. 7.14). From this, it was determined that the colostrum samples would have to be titrated out further to establish the optimum dilution, whereas the milk samples had a much lower level of detectable specific IgG. Six colostrum samples were titrated further, by fivefold dilution between 1 in 5 to 1 in 6,250, for the ELISA for IgG to the surface antigens, and twofold dilution between 1 in 5 and 1 in 160 for the ELISA for IgG to BoNT/C (Fig. 7.15).

On the basis of these preliminary experiments, all colostrum samples were diluted 1 in 100 for the screening ELISAs for *C. novyi* and *C. botulinum* type C surface antigens, and 1 in 20 for the screening ELISA for BoNT/C. Milk samples were diluted 1 in 10 for both ELISA assays. At these dilutions, the majority of sample had positive OD values, which fell on the linear part of the dilution curve.

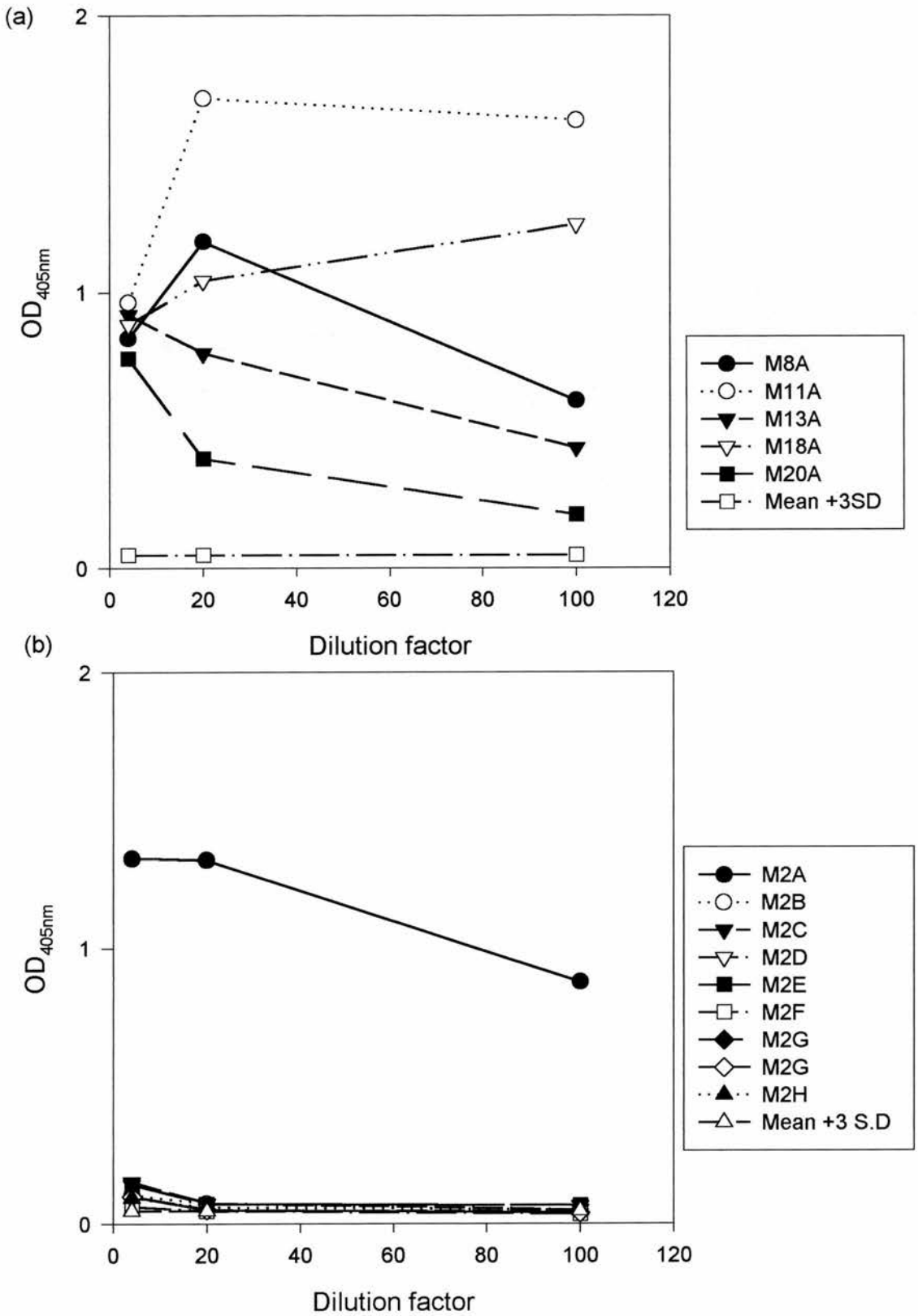
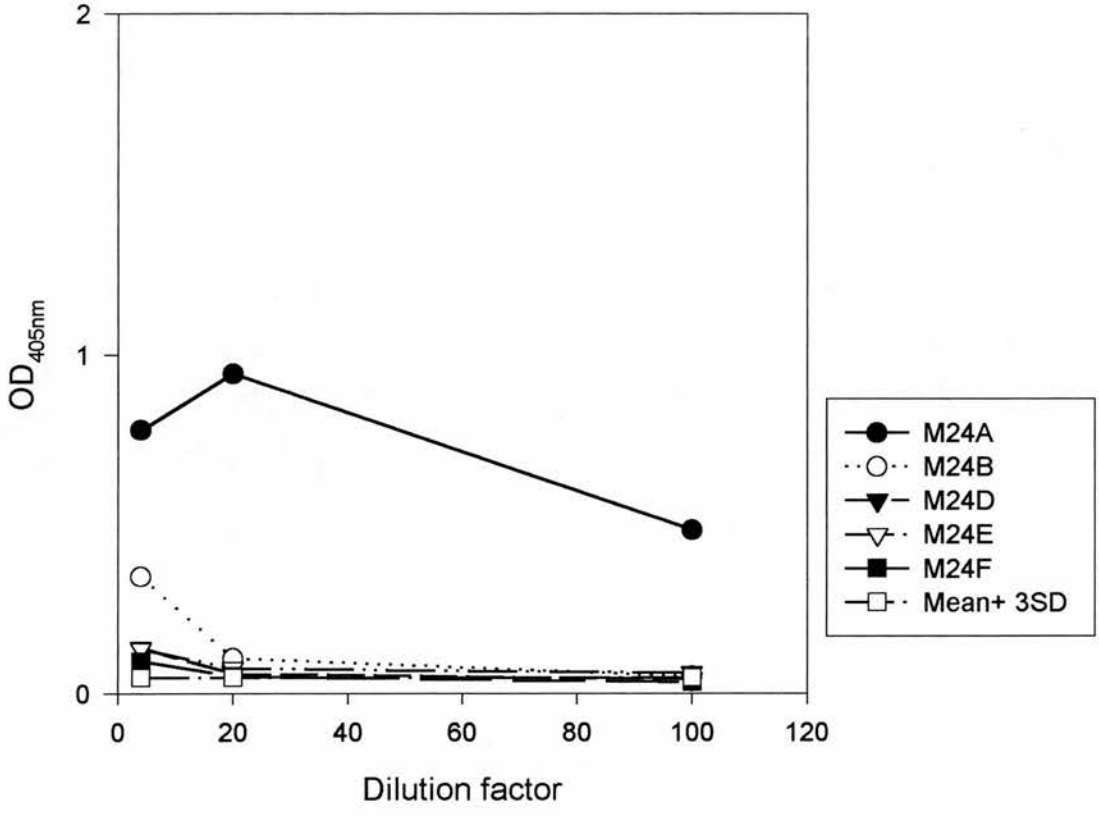


Figure 7.13: IgG in colostrum and milk samples to *C. novyi* type A surface antigens (a) colostrum samples and (b), (c) and (d) colostrum and milk samples diluted fivefold from 1 in 4 to 1 in 100. Samples with the suffix A are colostrum samples and samples with the suffix B, C, D etc are milk samples.

(c)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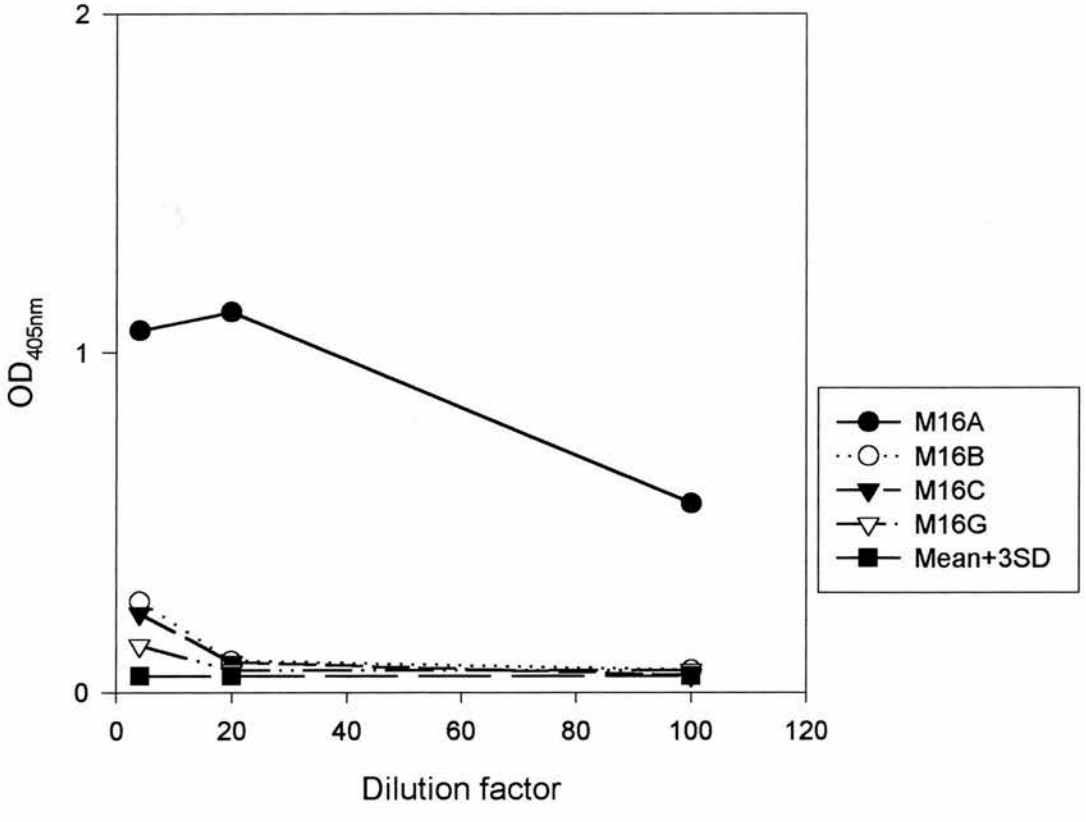
1
0
OD_{405nm}
0
20
40
60
80
100
120
Dilution factor
M24A
M24B
M24D
M24E
M24F
Mean+ 3SD

(d)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80
100
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Dilution factor
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M16B
M16C
M16G
Mean+3SD

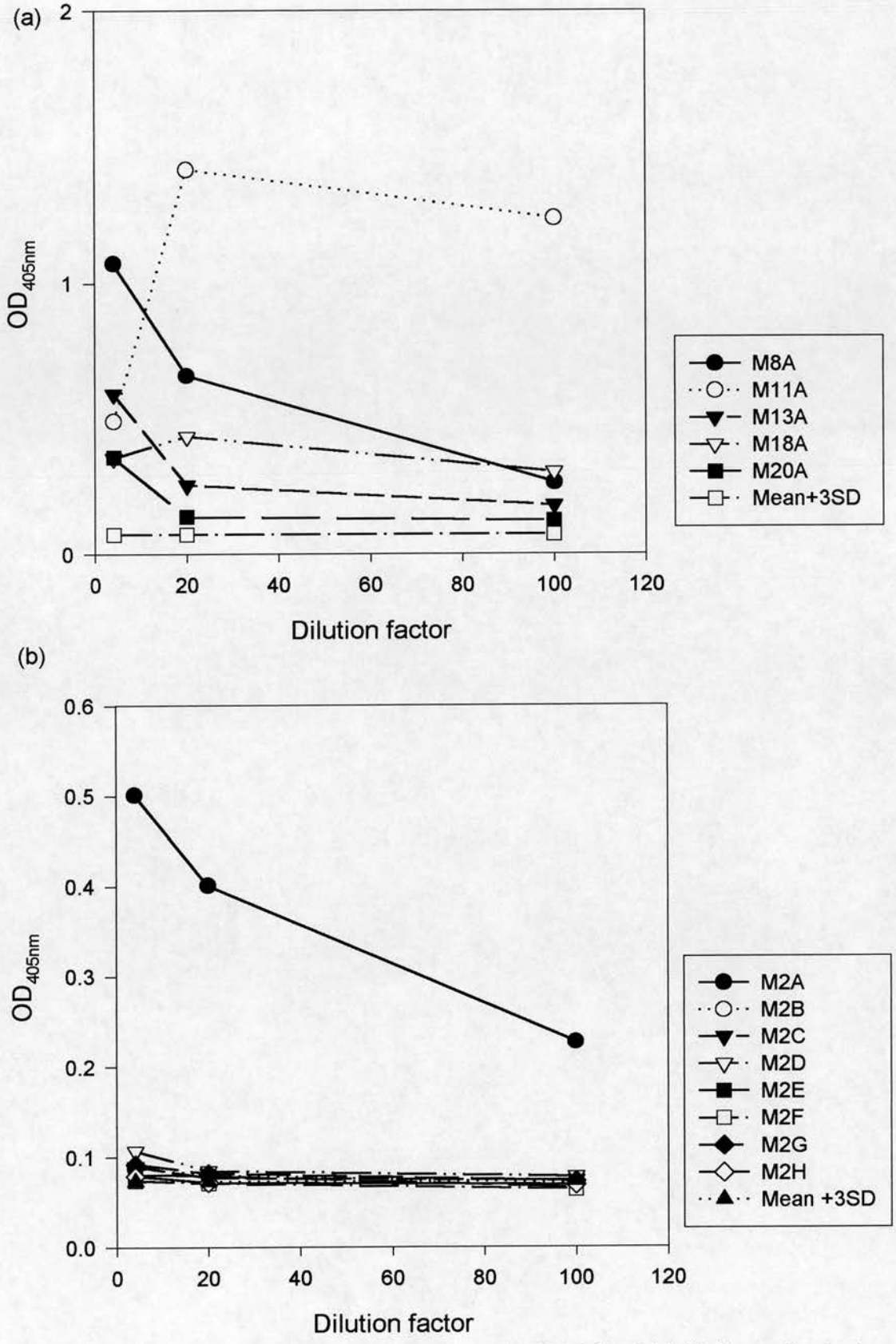
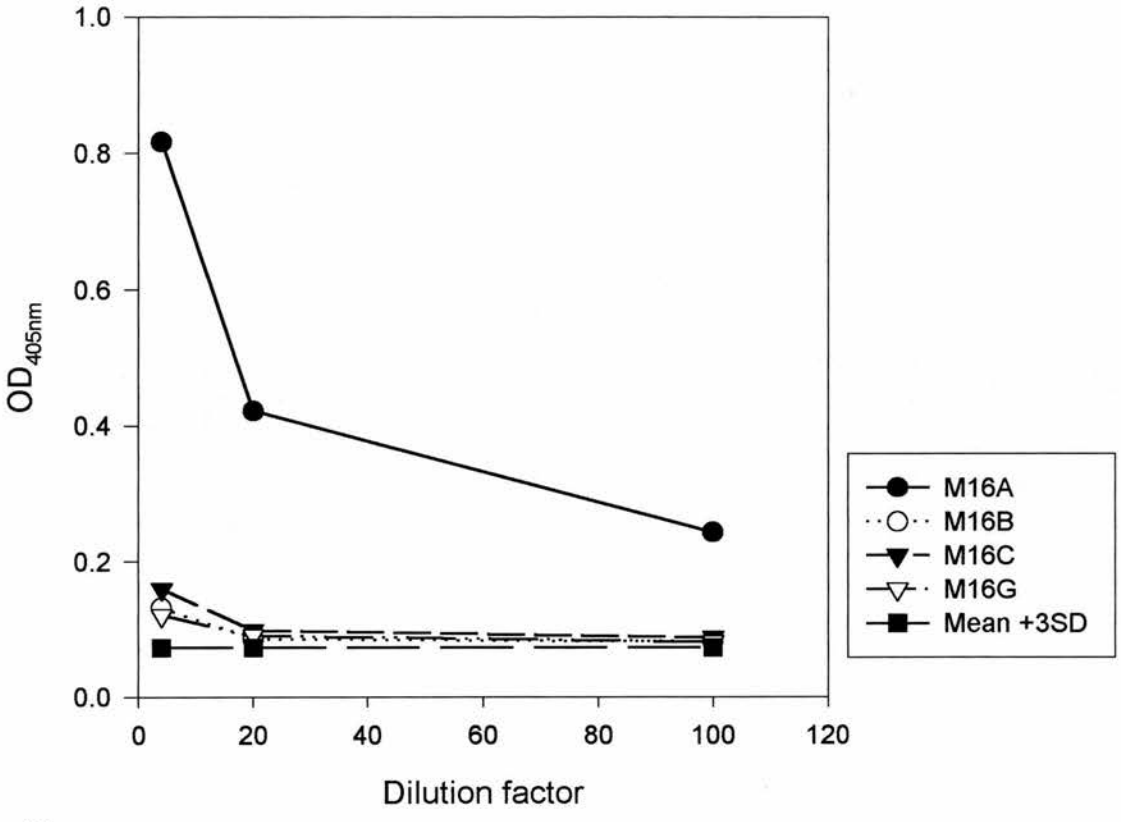
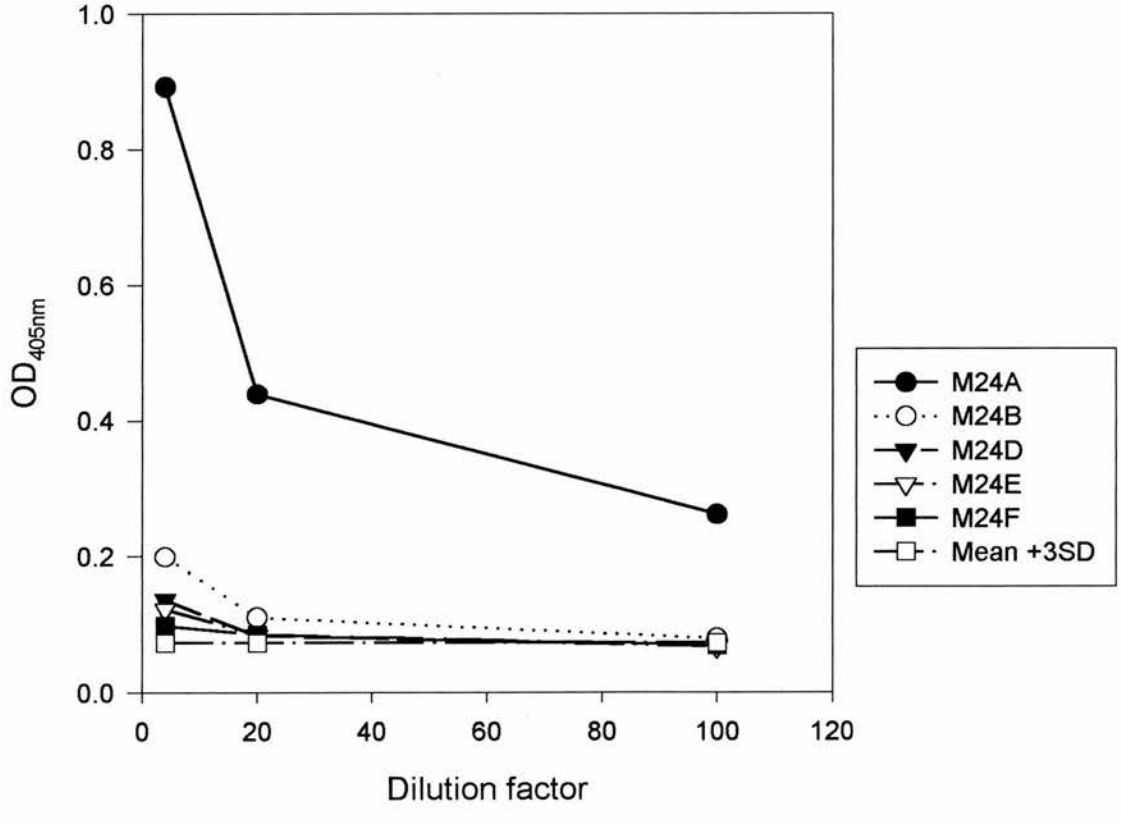


Figure 7.14: IgG in colostrum and milk to BoNT/C (a) fivefold dilutions of colostrum from 1 in 4 to 1 in 100 and (b), (c), and (d), fivefold dilutions of colostrum and milk samples between 1 in 4 and 1in 100.

(c)


1.0
0.8
0.6
0.4
0.2
0.0
OD405nm
0
20
40
60
80
100
120
Dilution factor
M16A
M16B
M16C
M16G
Mean +3SD

(d)


1.0
0.8
0.6
0.4
0.2
0.0
OD405nm
0
20
40
60
80
100
120
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M24A
M24B
M24D
M24E
M24F
Mean +3SD

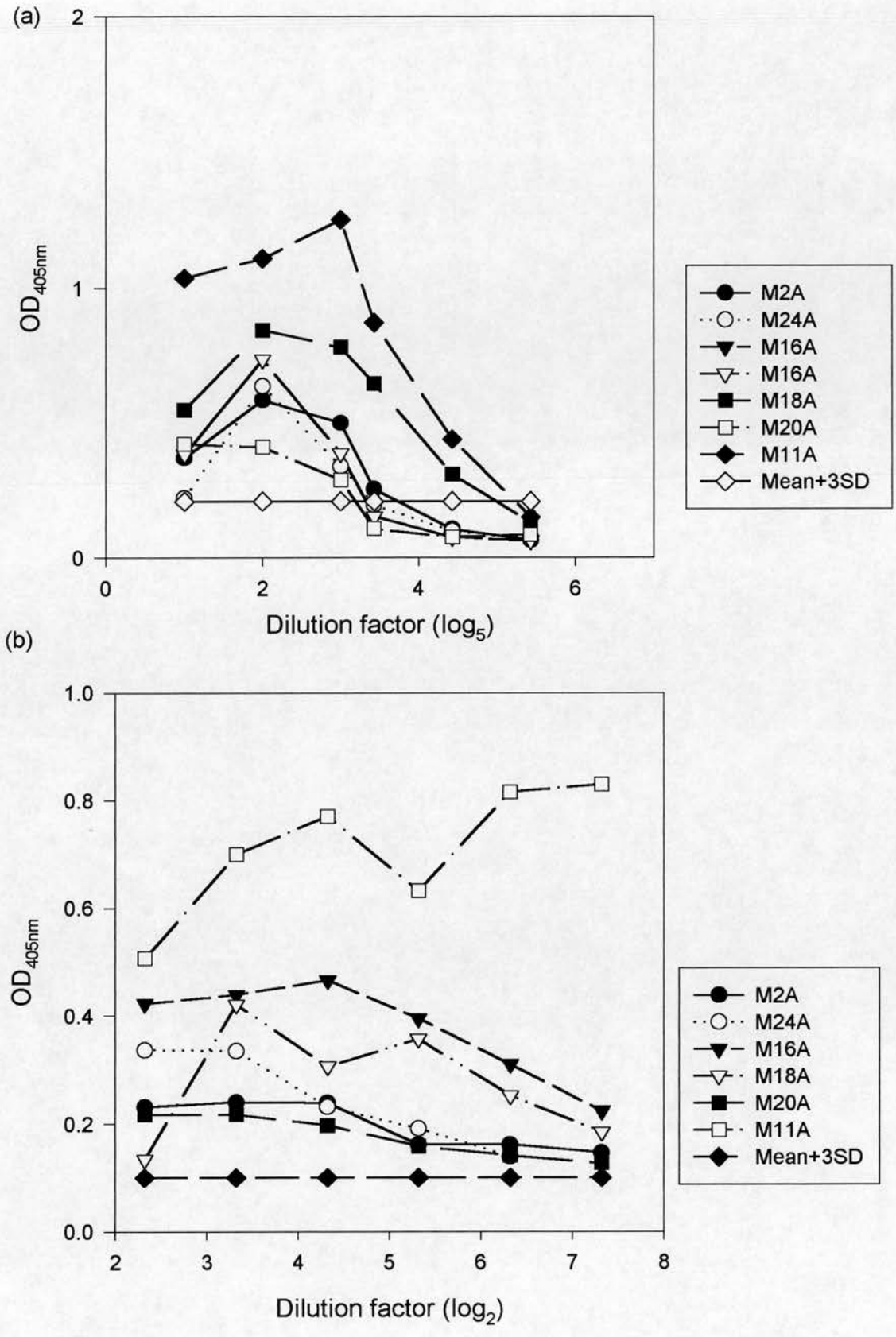


Figure 7.15: (a) IgG to *C. novyi* type A surface antigens in colostrum, samples diluted fivefold from 1in 5 to 1 in 6,250. (b) IgG to BoNT/C in colostrum, samples diluted twofold from 1 in 5 to 1 in 160.

Dilution of colostrum and milk samples for IgA screening assay

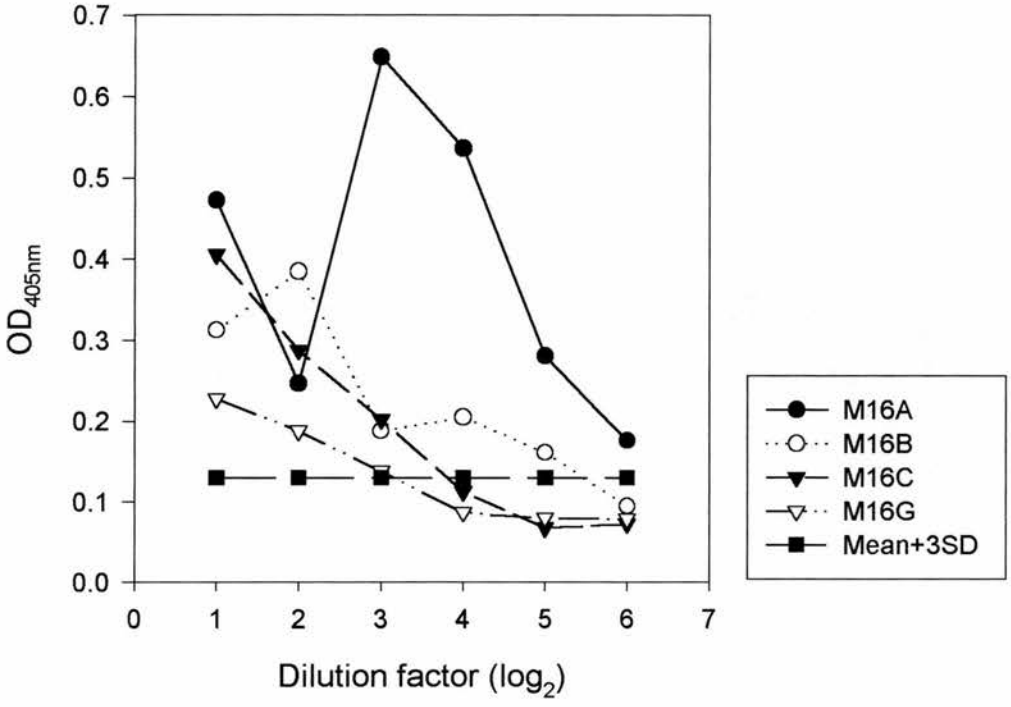
Preliminary assays were carried out to establish the optimal dilutions of colostrum and milk samples, for a screening assay to detect IgA to surface antigens and BoNT/C. Initially, three colostrum and 13 milk samples (collected at intervals over the suckling period) were diluted twofold between 1 in 2 and 1 in 64 (Fig. 7.16 and 7.17). Eight colostrum samples were titrated out further, with twofold dilutions from 1 in 5 to 1 in 160 (Fig. 7.18).

On the basis of these preliminary assays, it was decided to screen all colostrum samples at a 1 in 10 dilution for the ELISA for IgA to surface antigens and 1 in 5 dilution for the ELISA for IgA to BoNT/C. Milk samples were screened at a 1 in 4 dilution for the ELISAs for detecting IgA to both surface antigens and BoNT/C. At these dilutions, the majority of samples had positive OD values that also fell on the linear part of the dilution curve.

7.1.4 Calculation of total antibody levels in colostrum and milk by ELISA

Fivefold dilution of the colostrum samples between 1 in 100000 and 1 in 12500000 (Fig. 7.6), and milk samples between 1 in 1000 and 1 in 125000 (Fig. 7.8), produced a standard dilution curve for each sample. The OD of the dilution falling on the linear part of the dilution curve was chosen for calculation of total IgG in each sample. The equation of the line for the equine IgG standard was used to calculate the amount of IgG in each sample from the OD reading, taking into account the initial dilution factor.

(a)



(b)

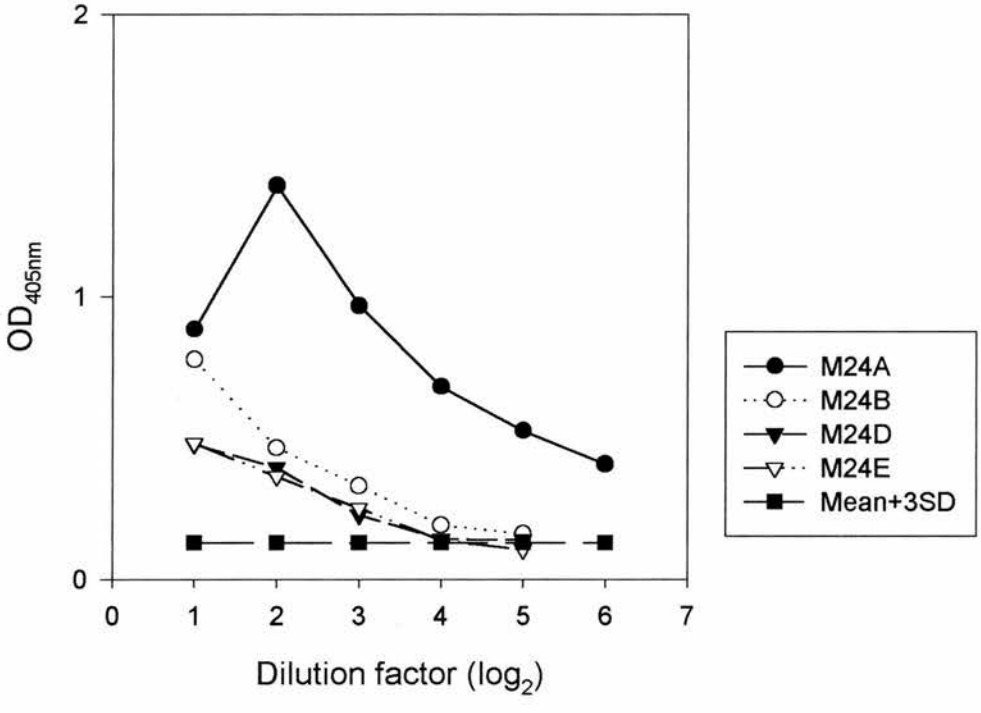
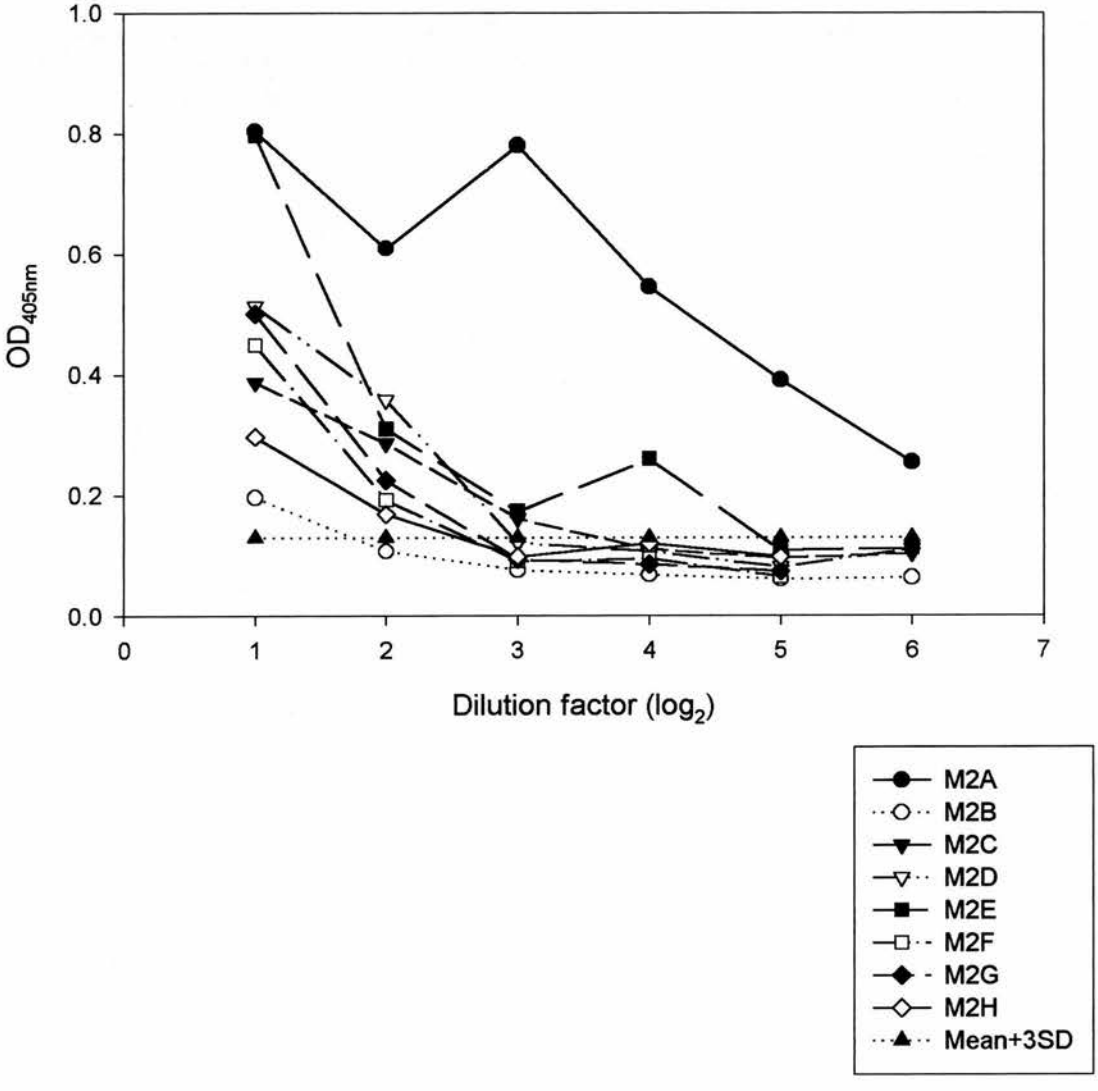
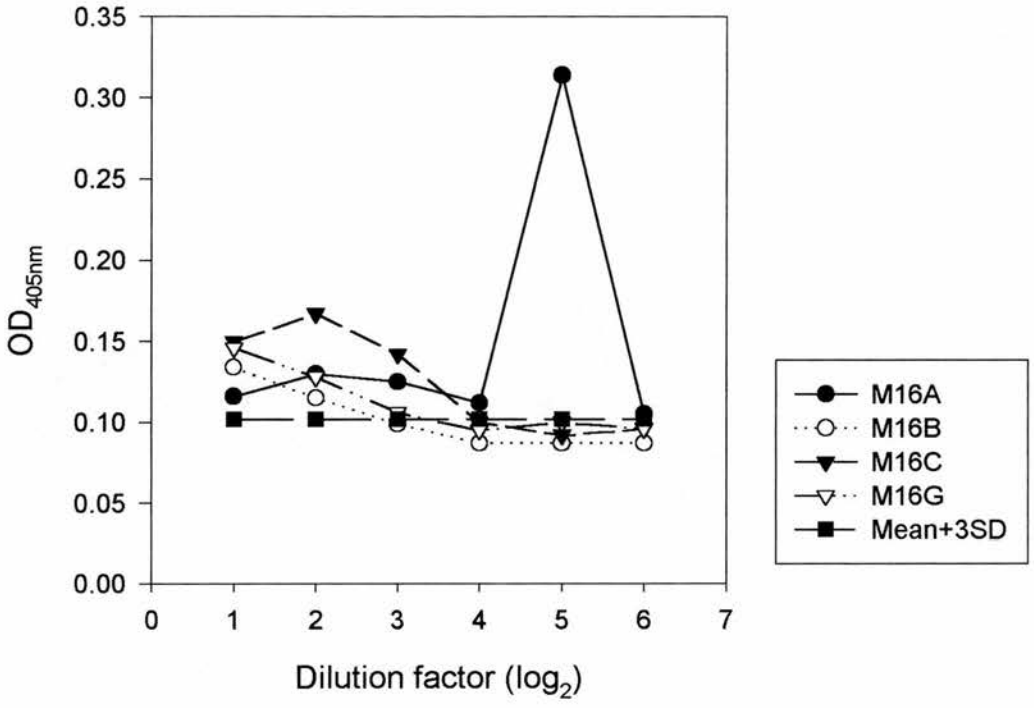


Figure 7.16: IgA to *C. novyi* type A surface antigens.
(a), (b), and (c) colostrum and milk samples diluted twofold from 1 in 2 to 1 in 64
The suffix of A denotes a colostrum sample e.g. M16A, and the suffix of B, C, D, E, F, G, or H, denotes a milk sample, e.g. M16B

(c)


1.0
0.8
0.6
0.4
0.2
0.0
OD405nm
0
1
2
3
4
5
6
7
Dilution factor (log2)
M2A
M2B
M2C
M2D
M2E
M2F
M2G
M2H
Mean+3SD

(a)



(b)

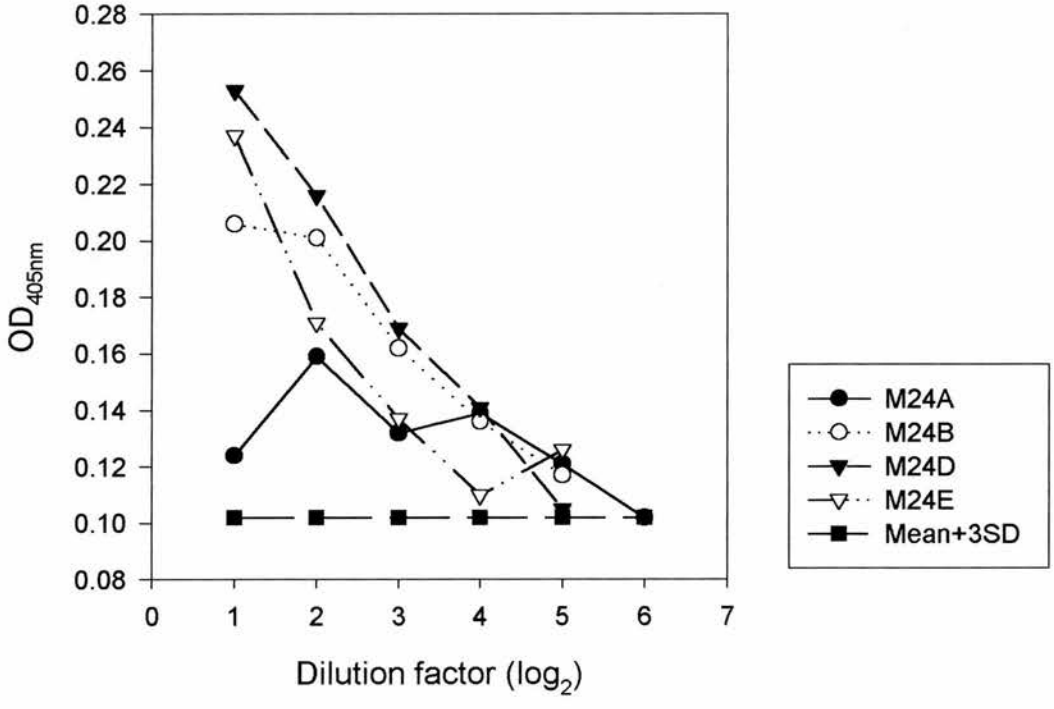
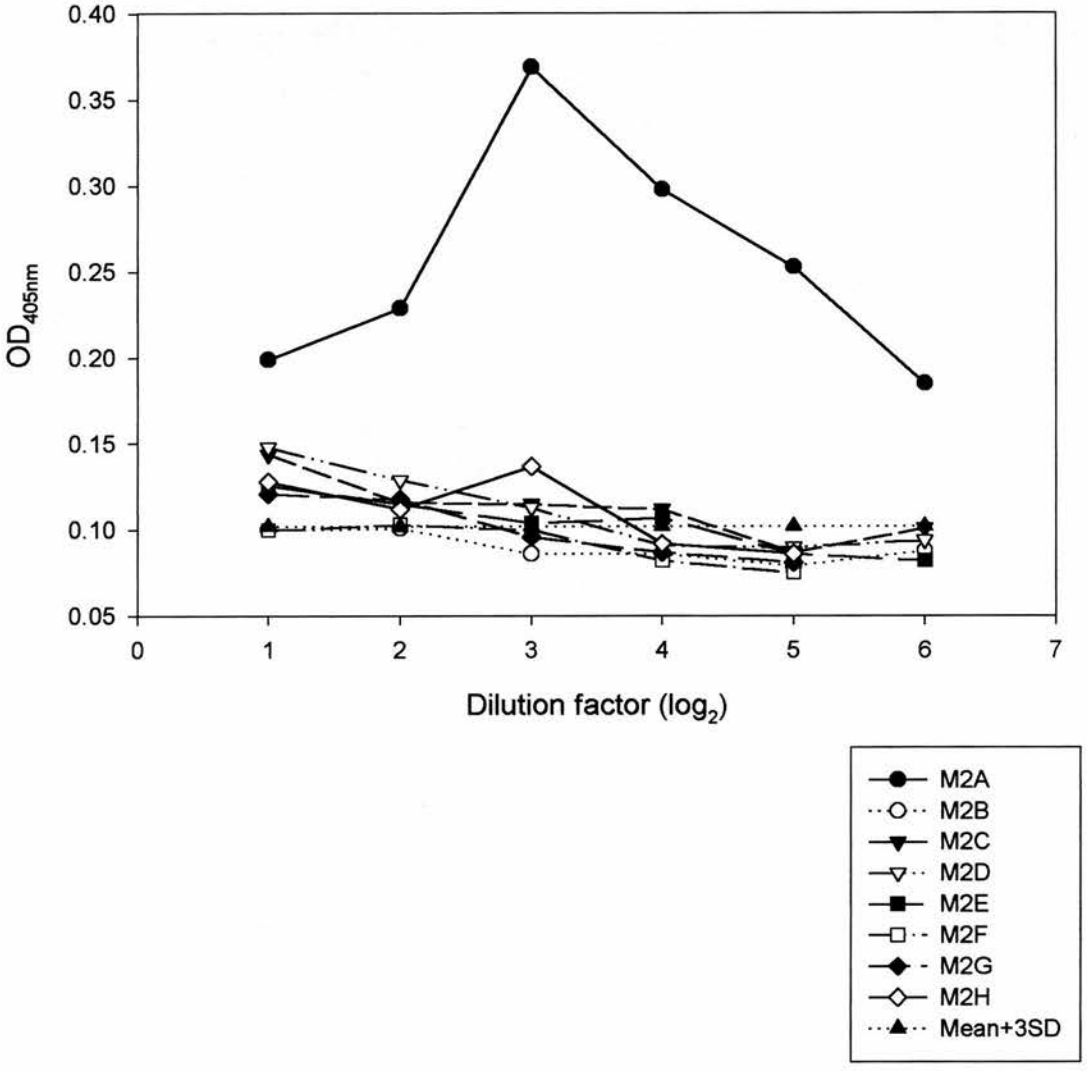


Figure 7.17: IgA to BoNT/C. (a), (b), and (c) colostrum and milk samples diluted twofold from 1in 2 to 1 in 64.

(c)


0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
OD405nm
0
1
2
3
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5
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M2D
M2E
M2F
M2G
M2H
Mean+3SD

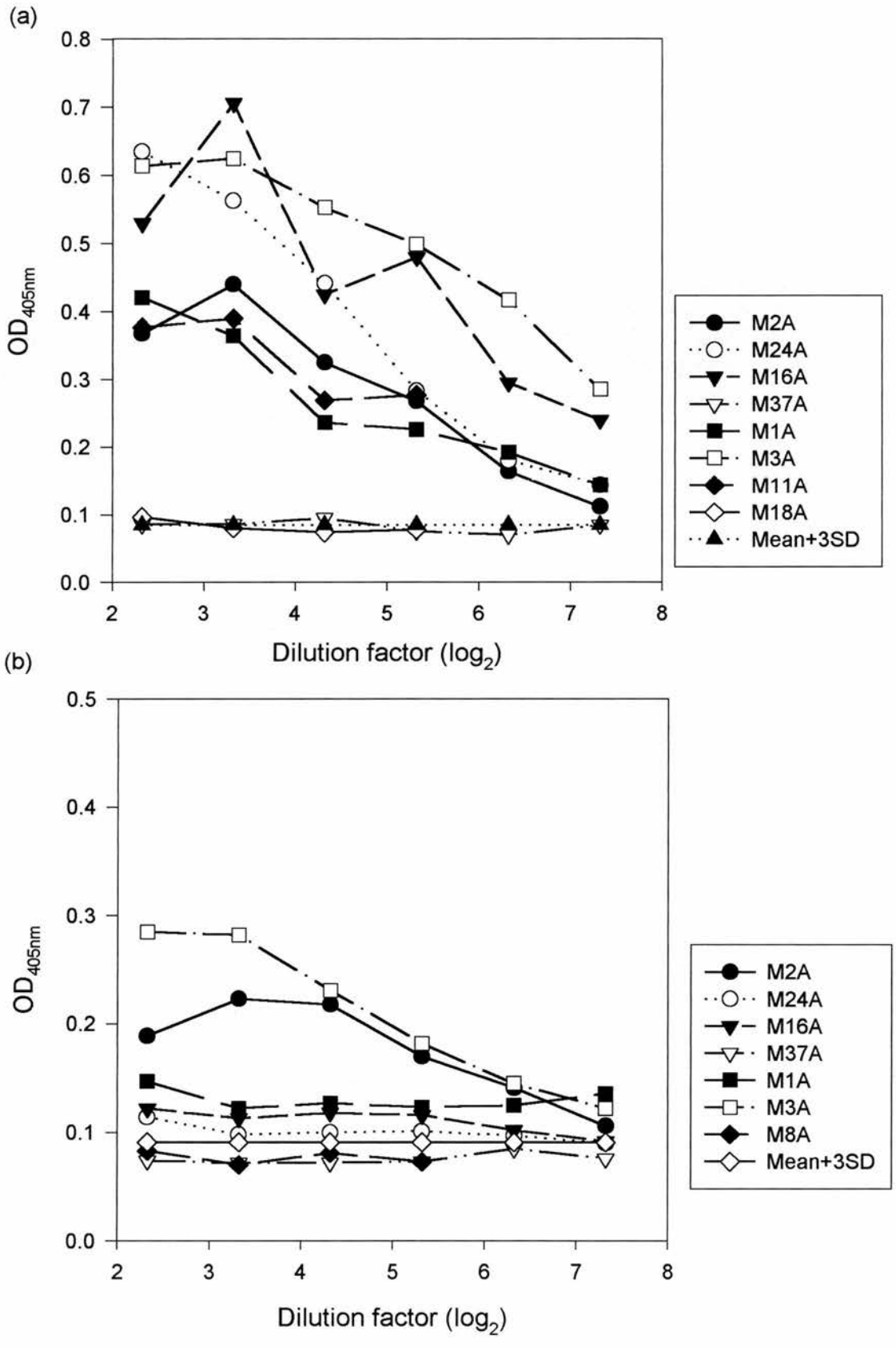


Figure 7.18: (a) IgA to *C. novyi* type A and (b) IgA to BoNT/C in colostrum Samples diluted twofold from 1 in 5 to 1 in 160

The mean value for total IgG in the 36 colostrum samples was found to be 116.2mg/ml, compared to a mean value of 2mg/ml in the milk samples (Table 7.2). Wide ranges of IgG levels were obtained. There was no significant difference in the levels of IgG in the colostrum or milk between grass sickness contact and non-contact groups.

For total IgA measurement, colostrum samples were diluted tenfold from 1 in 10 to 1 in 10,000 (Fig. 7.11d), and milk samples fivefold from 1 in 5 to 1 in 625 (Fig. 7.12). The OD of the dilution falling on the linear part of the dilution curve was chosen to calculate the total IgA present in each sample. The equation of the line of the IgA standard was used to calculate the amount of IgA in each sample, taking into account the initial dilution factor, and the $\log_2$ scale of the IgA standard.

The mean value for total IgA was 140 μg/ml for the colostrum samples and 20μg/ml for the milk samples (Table 7.2). There was no significant difference in total IgA content of the colostrum or milk, between the grass sickness contact and non-contact groups.

Table 7.2: The total IgG and IgA concentrations determined by ELISA in colostrum and milk samples

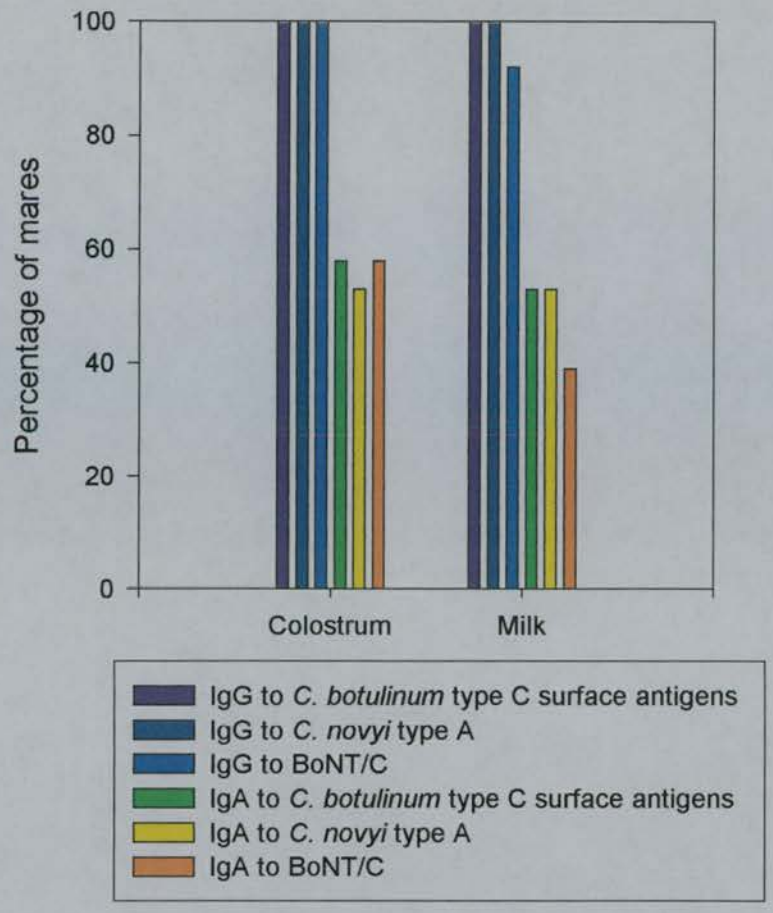
Statistics	Colostrum		Milk	
	Total IgG (mg/ml)	Total IgA (mg/ml)	Total IgG (mg/ml)	Total IgA (mg/ml)
Mean	116.2	1.4	2.0	0.02
Range	1.5 – 1110.9	0.002 – 33.4	0.6 – 4.3	0.002 – 0.2
S.D.	194	57	0.8	0.4

7.1.5 Detection of IgG and IgA, in colostrum and milk, to surface antigens and BoNT/C

All 36 colostrum samples had positive OD values for IgG to the surface antigens of *C. novyi* type A and *C. botulinum* type C and to BoNT/C, irrespective of whether the mare had been in contact with grass sickness previously or not (Fig. 7.19).

There was no significant difference between the levels of IgG to the surface antigens or to BoNT/C, detected in the colostrum of mares that had previously been in contact with grass sickness, compared to those that had not. This was the case irrespective of whether the results were expressed as corrected OD values or as OD values per mg of total IgG. Wide ranges of antibody levels were detected in the colostrum of mares in both groups (Table 7.3).

(a)



(b)

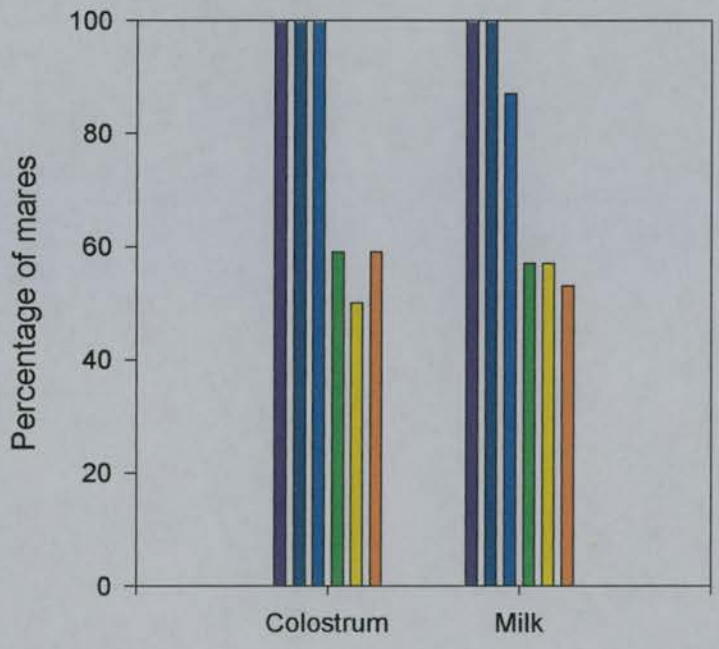


Figure 7.19: Percentage of mares with detectable IgG and IgA to *C. botulinum* type C surface antigens, *C. novyi* type A surface antigens and BoNT/C in colostrum and milk. (a) all mares, (b) mares in contact with grass sickness and (c) mares not in contact with grass sickness.

(c)

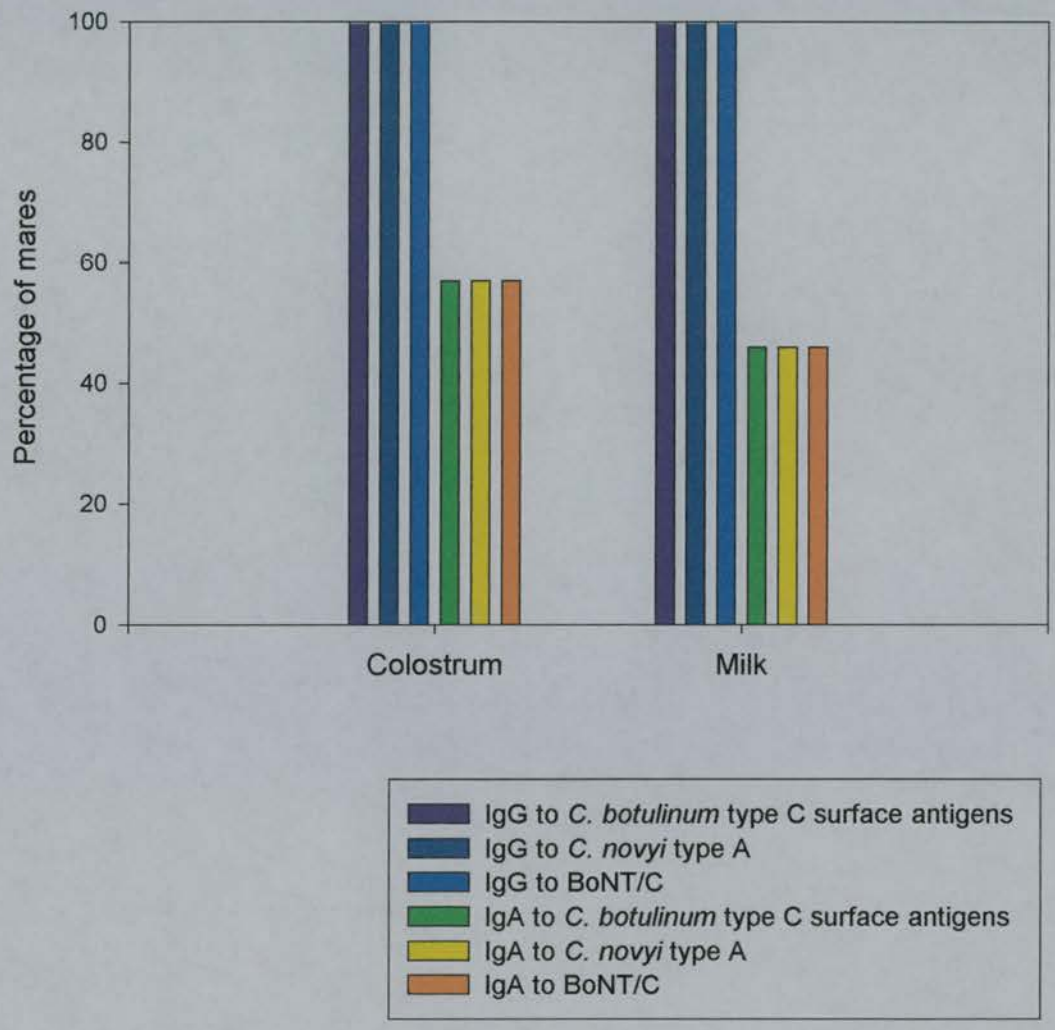

100
80
60
40
20
0
Percentage of mares
Colostrum
Milk
IgG to C. botulinum type C surface antigens
IgG to C. novyi type A
IgG to BoNT/C
IgA to C. botulinum type C surface antigens
IgA to C. novyi type A
IgA to BoNT/C

Table 7.3: IgG and IgA levels to *C. botulinum* type C and *C. novyi* type A surface antigens in the colostrum and milk of mares in contact and not in contact with equine grass sickness (EGS)

		Antibody levels OD_{405nm}			
		Colostrum		Milk	
Specific antibody	Statistics	Mares in contact with EGS	Mares not in contact with EGS	Mares in contact with EGS	Mares not in contact with EGS
IgG to BoNT/C	Mean	0.57	0.66	0.14	0.16
	Range	0.12-1.34	0.12-2.0	0.08-0.31	0.09-0.53
	S.D.	0.43	0.54	0.06	0.12
IgG to *C. bot.* type C surface antigens	Mean	0.95	1.03	0.33	0.38
	Range	0.19-1.68	0.38-1.82	0.14-0.58	0.22-0.62
	S.D.	0.53	0.48	0.13	0.12
IgG to *C. novyi* type A surface antigens	Mean	0.4	0.41	0.24	0.26
	Range	0.09-0.97	0.08-0.95	0.12-0.43	0.18-0.35
	S.D.	0.28	0.25	0.11	0.06
IgA to BoNT/C	Mean	0.09	0.14	0.11	0.14
	Range	0.04-0.24	0.04-0.38	0.04-0.37	0.04-0.36
	S.D.	0.06	0.11	0.08	0.11
IgA to *C. bot.* type C surface antigens	Mean	0.3	0.47	0.26	0.33
	Range	0.03-1.25	0.03-1.2	0.03-0.78	0.03-1.4
	S.D.	0.32	0.45	0.24	0.42
IgA to *C. novyi* type A surface antigens	Mean	0.27	0.39	0.19	0.23
	Range	0.03-1.47	0.03-1.44	0.03-0.53	0.03-1.1
	S.D.	0.35	0.42	0.17	0.32

Specific IgA was detected, by ELISA, in 58% (21/36) of colostrum samples both to the surface antigens of *C. botulinum* type C and to BoNT/C (Fig. 7.19a). 59% (13/22) of mares that had been in contact with grass sickness had detectable IgA in the colostrum to these specific antigens (Fig. 7.19b), compared with the colostrum of 57% (8/14) of mares that had not been in contact with grass sickness (Fig. 7.19c).

IgA was detected to the surface antigens of *C. novyi* type A in 53% (19/36) of colostrum samples. 50% (11/22) of mares in contact with grass sickness, and 57% (8/14) of mares not in contact, had detectable IgA to *C. novyi* in the colostrum.

There was no significant difference between the levels of IgA detected to either the surface antigens or to BoNT/C, between the mares that had been in contact with grass sickness and those that had not (Table 7.3). A wide range of antibody levels was detected in the colostrum of mares in both these groups (Table 7.3).

All the colostrum samples that were negative for IgA to BoNT/C were also negative for IgA to *C. botulinum* type C surface antigens and *C. novyi* type A surface antigens, with two exceptions from the "contact" group. M18A had detectable IgA to BoNT/C but not to the surface antigens, and M25A had detectable IgA to the surface antigens but not to BoNT/C. Another colostrum sample from the "contact" group, M35A, did not have detectable IgA to *C. novyi* type A surface antigens but did have detectable antibodies to the surface antigens of *C. botulinum* type C and BoNT/C.

A positive correlation was seen in the colostrum between the levels of IgG to BoNT/C and the levels of IgG to *C. botulinum* type C surface antigens (R=0.75) (Fig. 7.20a). A positive correlation was also seen between the levels of IgA in colostrum to BoNT/C and to *C. botulinum* type C surface antigens (R=0.83) (Fig. 7.21a). A greater level of correlation was observed between the IgG and IgA responses to these antigens when the OD values were expressed as OD/mg total IgG (R=0.93) or as OD/μg total IgA (R=0.84) (Fig. 7.20b and 7.21b).

A greater correlation between the IgG response to BoNT/C and surface antigens was seen in the colostrum of mares in contact with grass sickness (R=0.87), than those not in contact with grass sickness (R=0.6) (or R=0.97 and R=0.81 respectively when expressed as OD/mg total IgG) (Fig. 7.20c-f and Table 7.4).

A greater level of correlation was also observed between the IgA response to BoNT/C and surface antigens in the colostrum of mares in contact with grass sickness (R=0.94), than those not in contact (R=0.77) (or R=0.91 and R= 0.78 respectively when expressed as OD/μg total IgA) (Fig. 7.21 c-f and Table 7.4).

IgG to *C. botulinum* type C surface antigens was detected in all 36 milk samples (Fig. 7.19a). IgG to BoNT/C was detected in 92% (33/36) of the milk samples (Fig. 7.19a). The three milk samples that did not have detectable IgG to BoNT/C were from mares that had been in contact with grass sickness, and two out of the three did not have detectable IgA to BoNT/C or the surface antigens in both colostrum and milk.

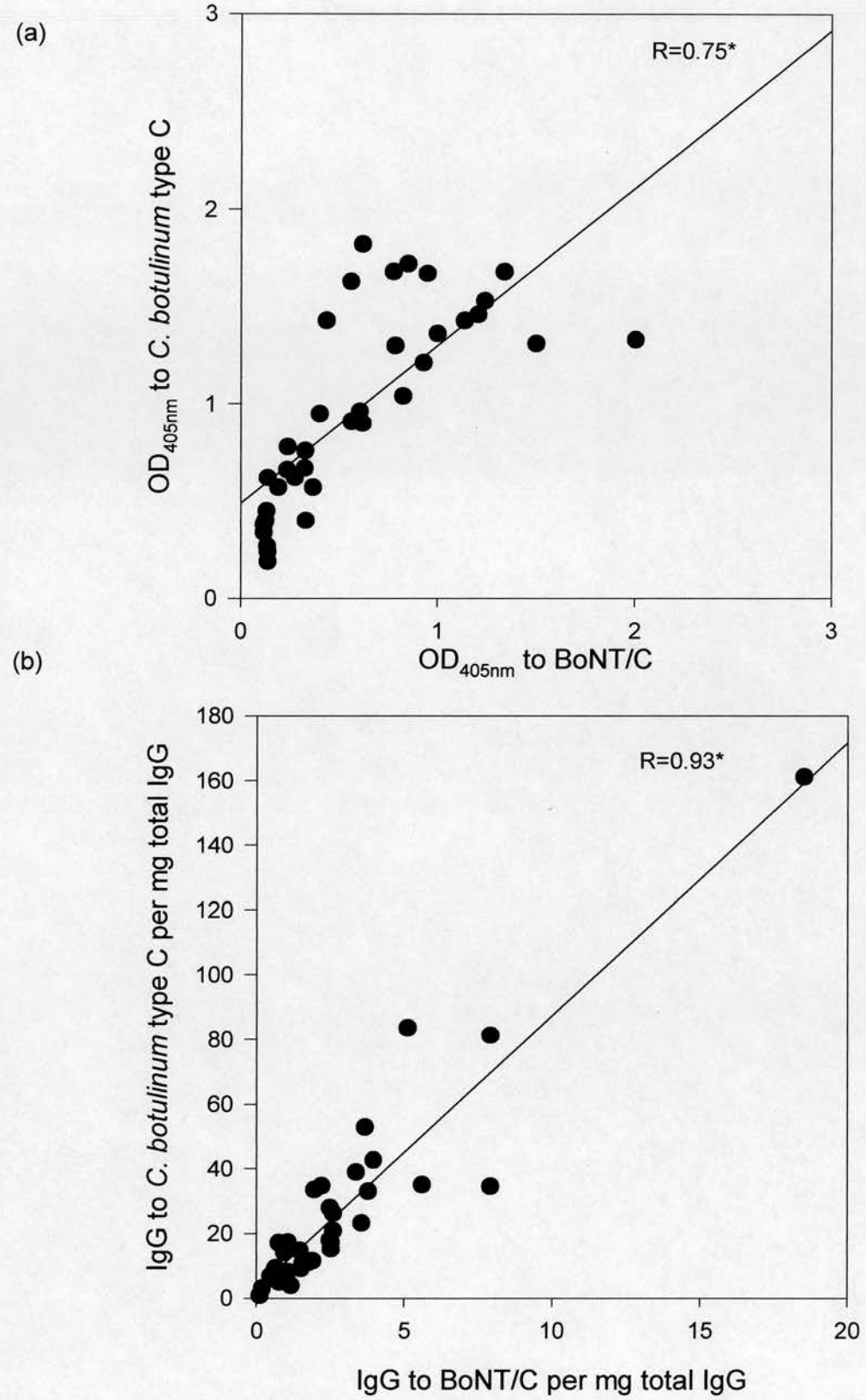
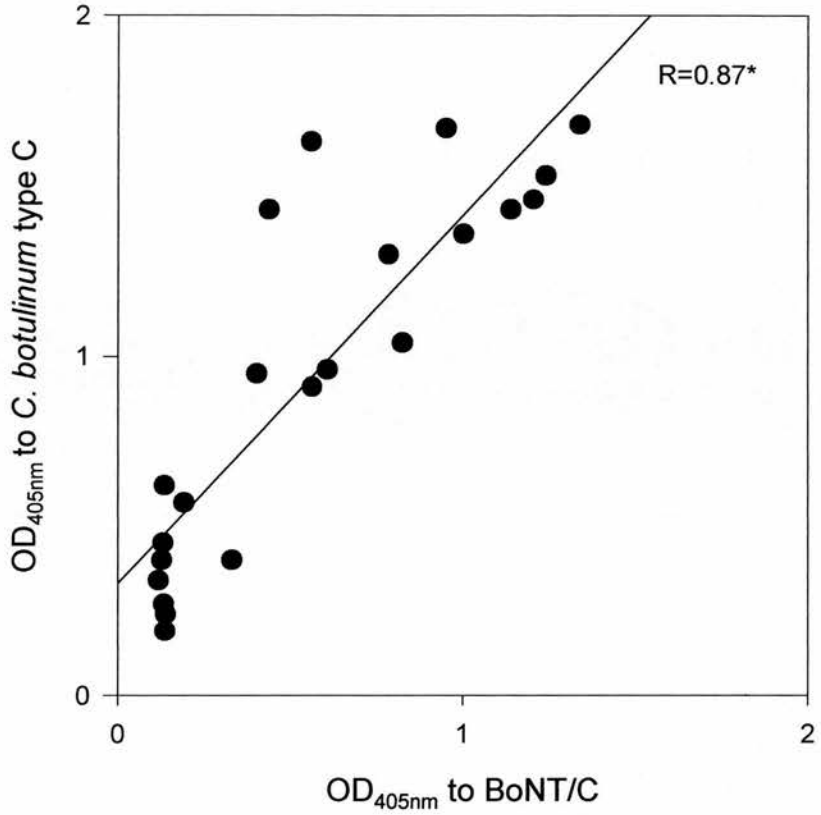
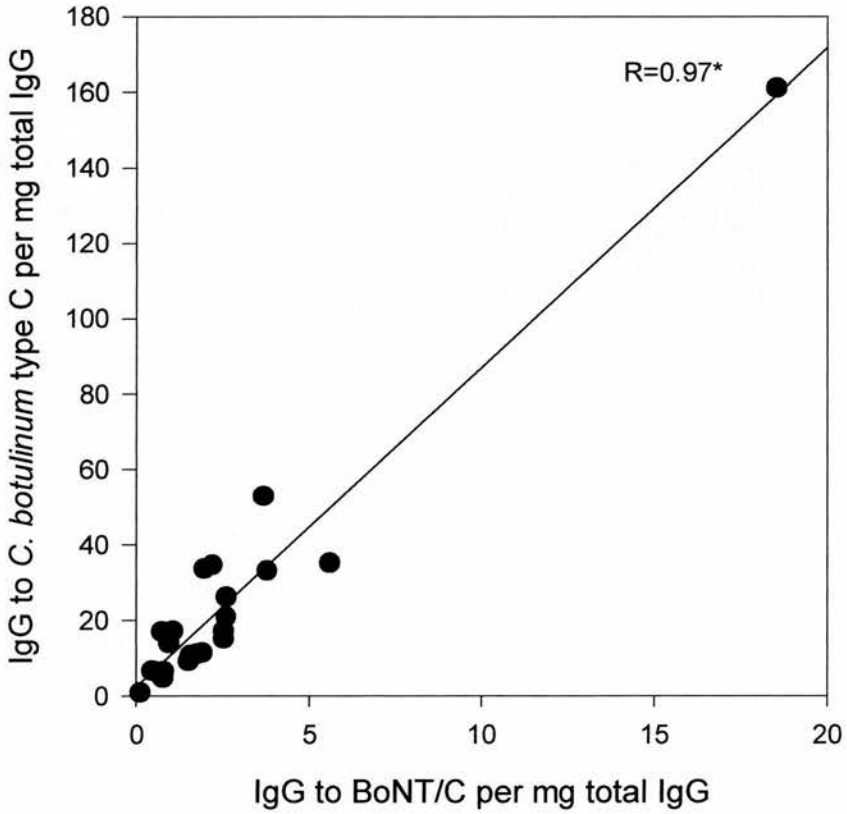


Figure 7.20: IgG to BoNT/C against IgG to *C. botulinum* type C surface antigens in colostrum (a), (c) and (e) levels of IgG expressed as OD readings and (b), (d) and (f) levels of specific IgG expressed as OD/mg total IgG. (a) and (b) all mares, (c) and (d) mares in contact with grass sickness, and (e) and (f) mares not in contact with grass sickness.
*Correlation is significant at the 0.01 level.
**Correlation is significant at the 0.05 level.

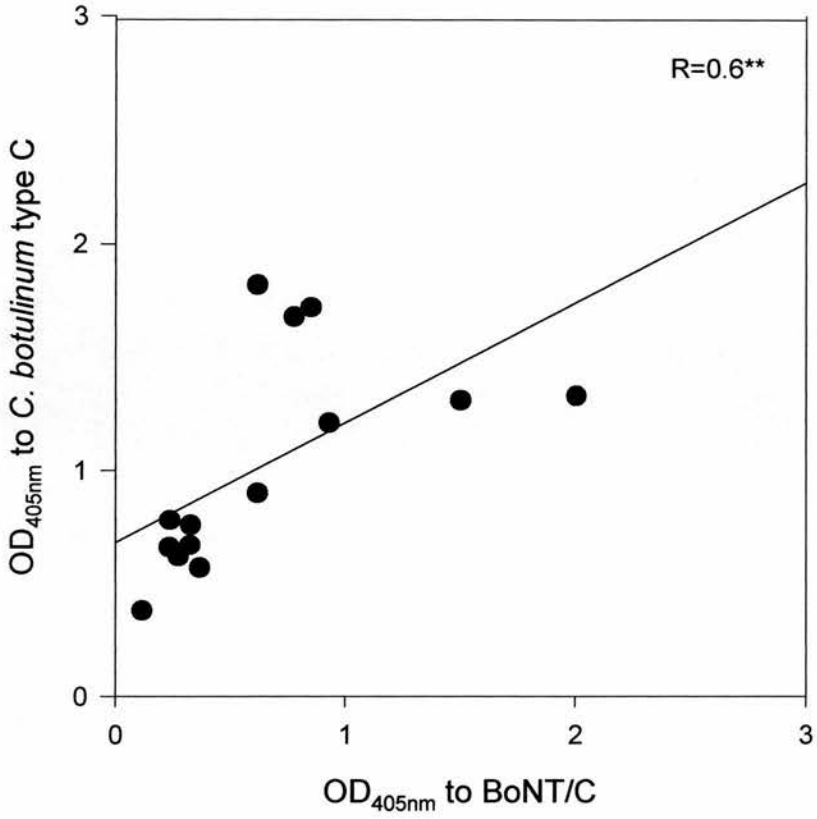
(c)


R=0.87*
OD_{405nm} to *C. botulinum* type C
OD_{405nm} to BoNT/C
0
1
2

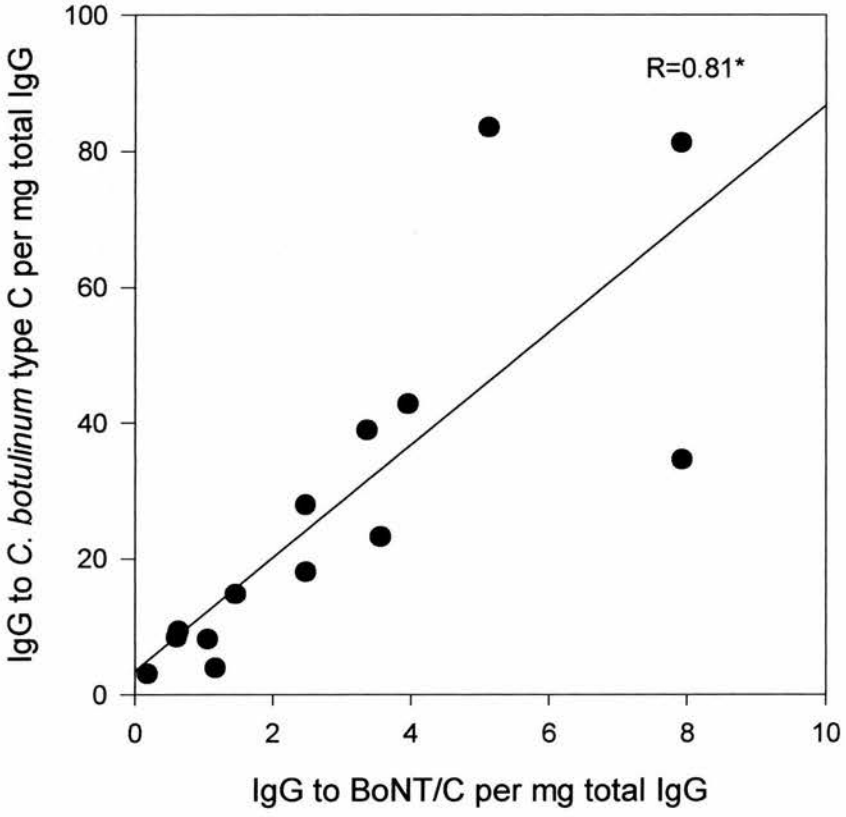
(d)


R=0.97*
IgG to *C. botulinum* type C per mg total IgG
IgG to BoNT/C per mg total IgG
0
20
40
60
80
100
120
140
160
180
0
5
10
15
20

(e)


R=0.6**
OD_{405nm} to *C. botulinum* type C
OD_{405nm} to BoNT/C
0
1
2
3

(f)


R=0.81*
IgG to *C. botulinum* type C per mg total IgG
IgG to BoNT/C per mg total IgG
0
20
40
60
80
100
0
2
4
6
8
10

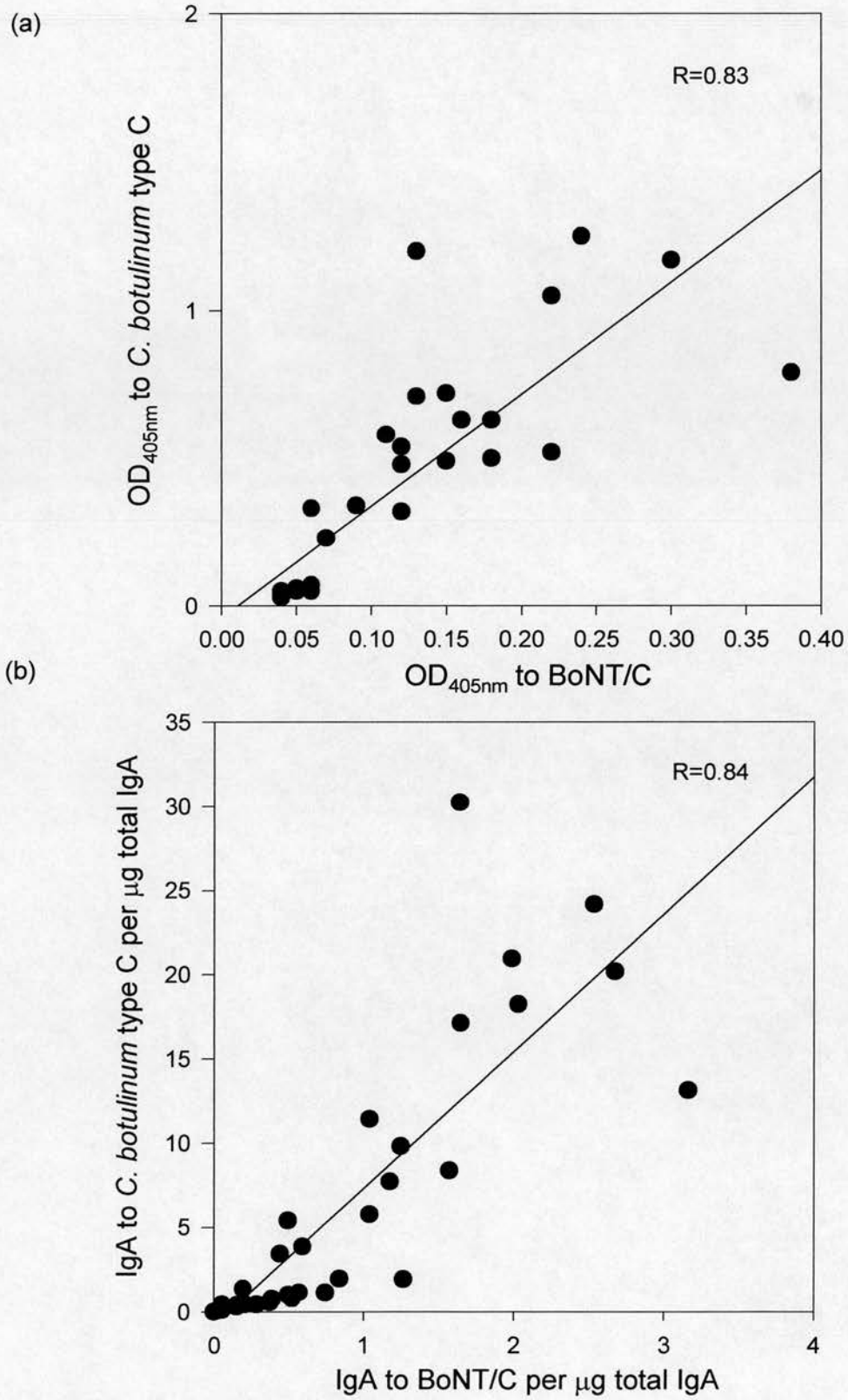
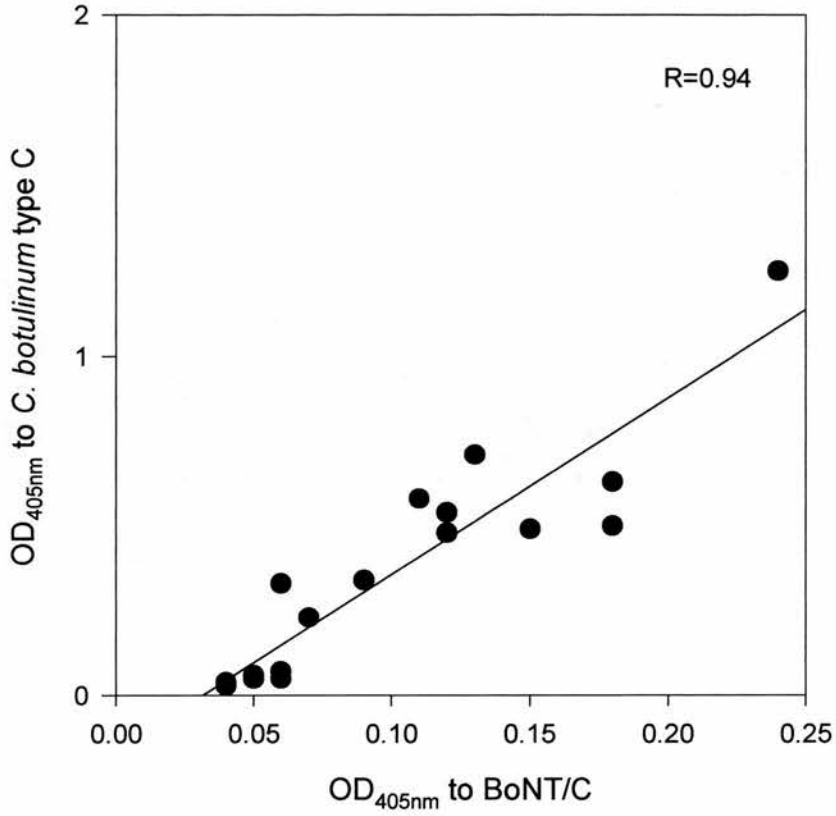
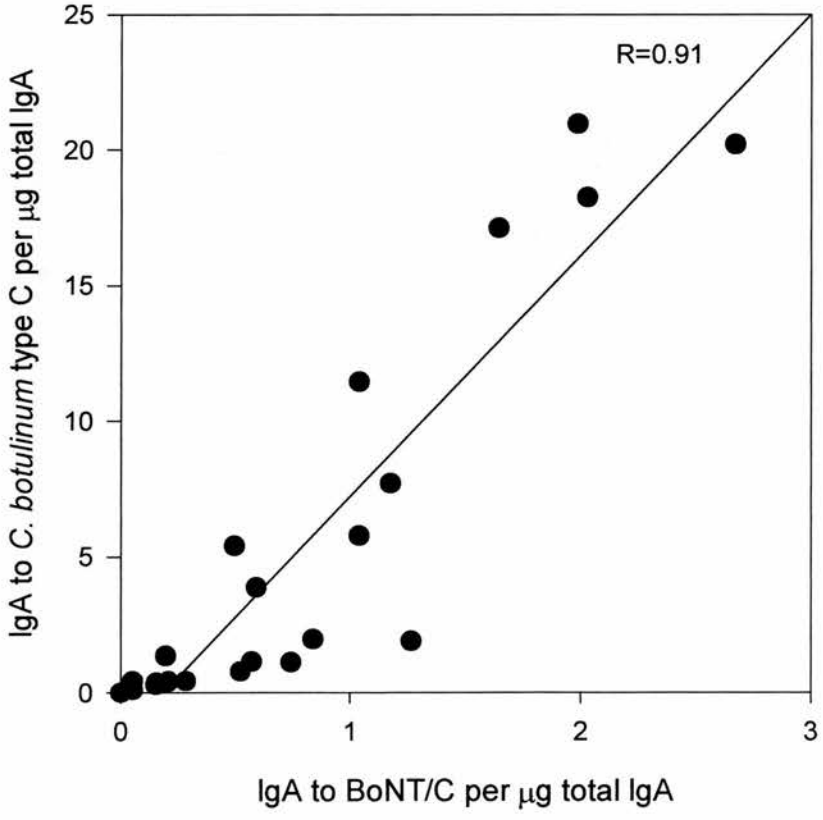


Figure 7.21: IgA to BoNT/C against IgA to *C. botulinum* type C surface antigens in colostrum (a), (c) and (e) IgA expressed as OD readings, and (b), (d), and (f), specific IgA expressed as OD/μg total IgA. (a) and (b) all mares, (c) and (d) mares in contact with grass sickness, and (e) and (f), mares not in contact with grass sickness. Correlation is significant at the 0.01 level.

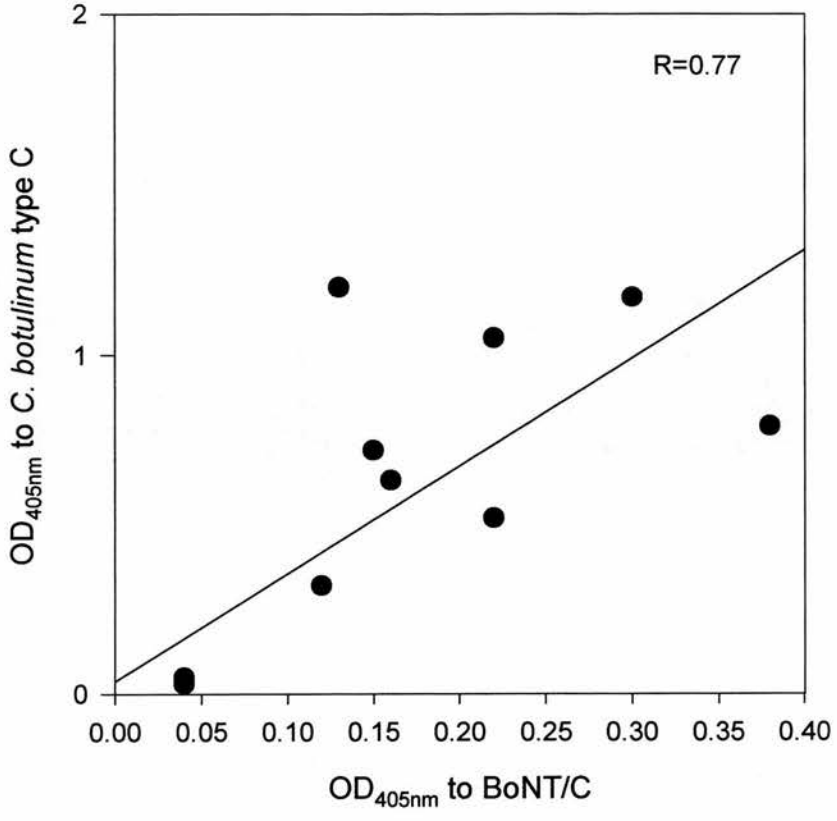
(c)


R=0.94
OD_{405nm} to *C. botulinum* type C
OD_{405nm} to BoNT/C
0
1
2
0.00
0.05
0.10
0.15
0.20
0.25

(d)


R=0.91
IgA to *C. botulinum* type C per μg total IgA
IgA to BoNT/C per μg total IgA
0
5
10
15
20
25
0
1
2
3

(e)


R=0.77
OD_{405nm} to *C. botulinum* type C
OD_{405nm} to BoNT/C
0
1
2
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40

(f)

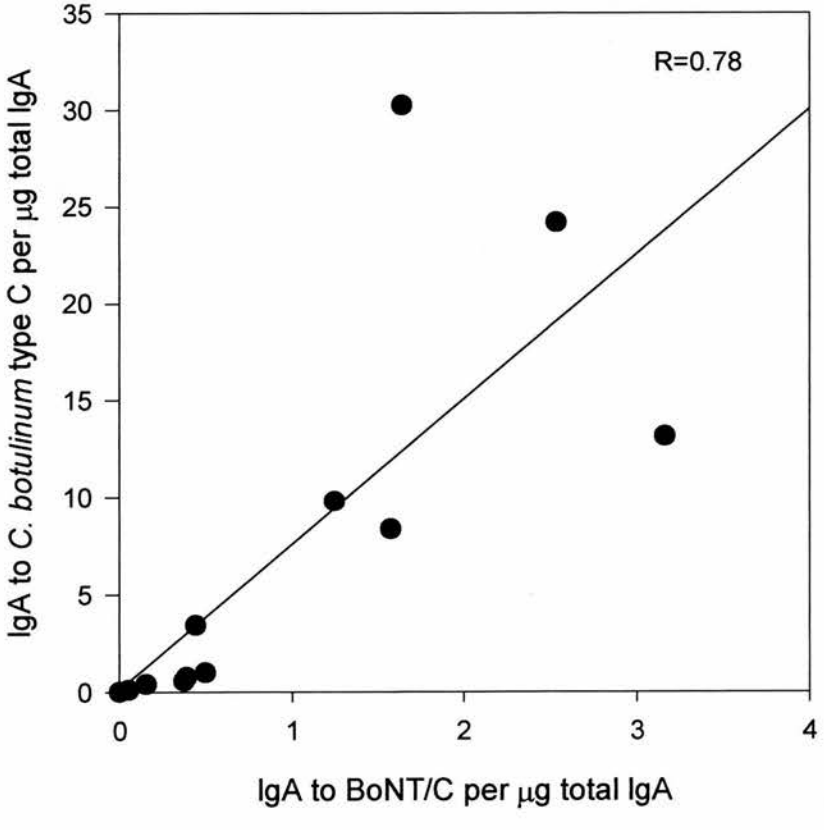

R=0.78
IgA to *C. botulinum* type C per μg total IgA
IgA to BoNT/C per μg total IgA
0
5
10
15
20
25
30
35
0
1
2
3
4

Table 7.4: Correlation between the immune response to *C. botulinum* type C surface antigens and BoNT/C in colostrum and milk.

	Colostrum			Milk		
***C. botulinum* type C surface antigens vs. BoNT/C**	**All mares (n=36)**	**Mares in contact with EGS (n=22)**	**Mares not in contact with EGS (n=14)**	**All mares (n=36)**	**Mares in contact with EGS (n=23)**	**Mares not in contact with EGS (n=13)**
IgG per mg total IgG	R=0.93*	R=0.97*	R=0.81*	R=0.47*	R=0.55*	R=0.41
IgG as OD	R=0.75*	R=0.87*	R=0.60**	R=0.11	R=0.25	R=0.1
IgA per μg total IgA	R=0.84*	R=0.91*	R=0.78*	R=0.84*	R=0.89*	R=0.73*
IgA as OD	R=0.83*	R=0.94*	R=0.77*	R=0.75*	R=0.81*	R=0.71*

* correlation is significant at the 0.01 level
** correlation is significant at the 0.05 level

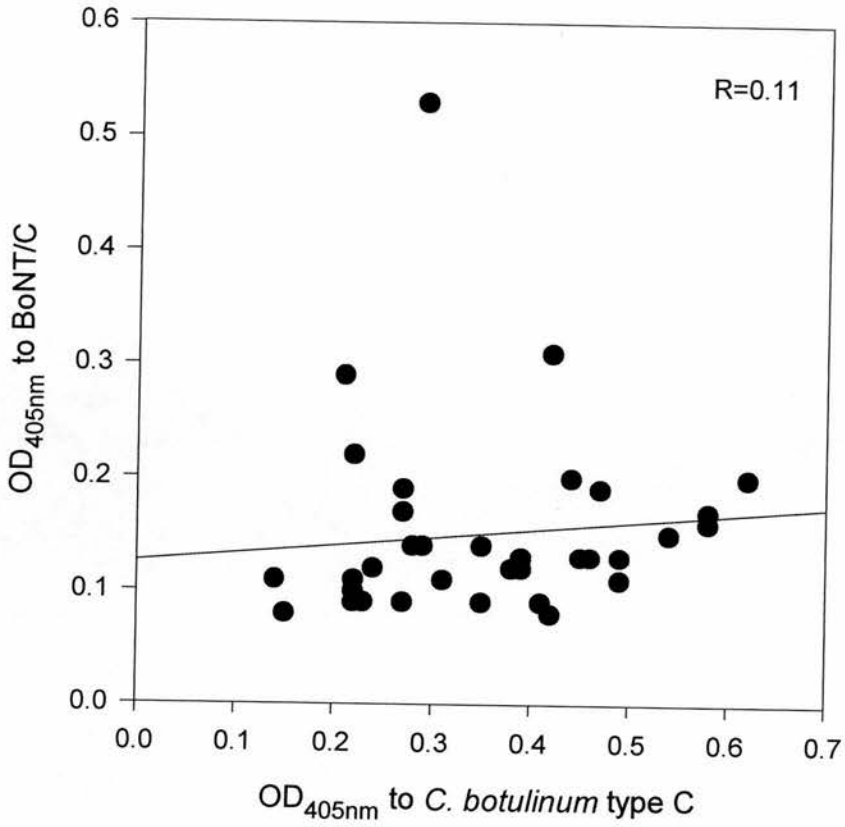
There were no significant differences between IgG levels in milk to BoNT/C or to surface antigens between mares that had been in contact with grass sickness and those that had not. Wide ranges in levels of antibodies were seen in both groups (Table 7.3).

IgA was detected to *C. botulinum* type C surface antigens in 53% (19/36) of milk samples and to BoNT/C in 39% (14/36) of milk samples (Fig. 7.19a). IgA was detected to surface antigens in 57% of the milk samples from mares that had been in contact with grass sickness and to BoNT/C in 35% of these milk samples (Fig. 7.19b). 46% of milk samples from mares not in contact with grass sickness had detectable IgA to both the surface antigens and BoNT/C (Fig. 7.19c).

All of the 17 samples, which did not have detectable IgA to surface antigens in milk also did not have detectable IgA to BoNT/C in the milk. 15 of these samples were from mares that also did not have detectable IgA in the colostrum to surface antigens or BoNT/C.

The correlation between IgG to BoNT/C and IgG to surface antigens observed in milk samples (R=0.47) was not as great as that observed in colostrum (R=0.93). Expression of results as specific IgG/mg total IgG, increased the level of correlation from R=0.11 to R=0.5 (Fig. 7.22a and 7.22b). Greater correlation between IgG to BoNT/C and surface antigens was observed in milk from mares which had been in contact with EGS (R=0.62) compared to those which had not (R=0.4) (Table 7.4).

(a)



(b)

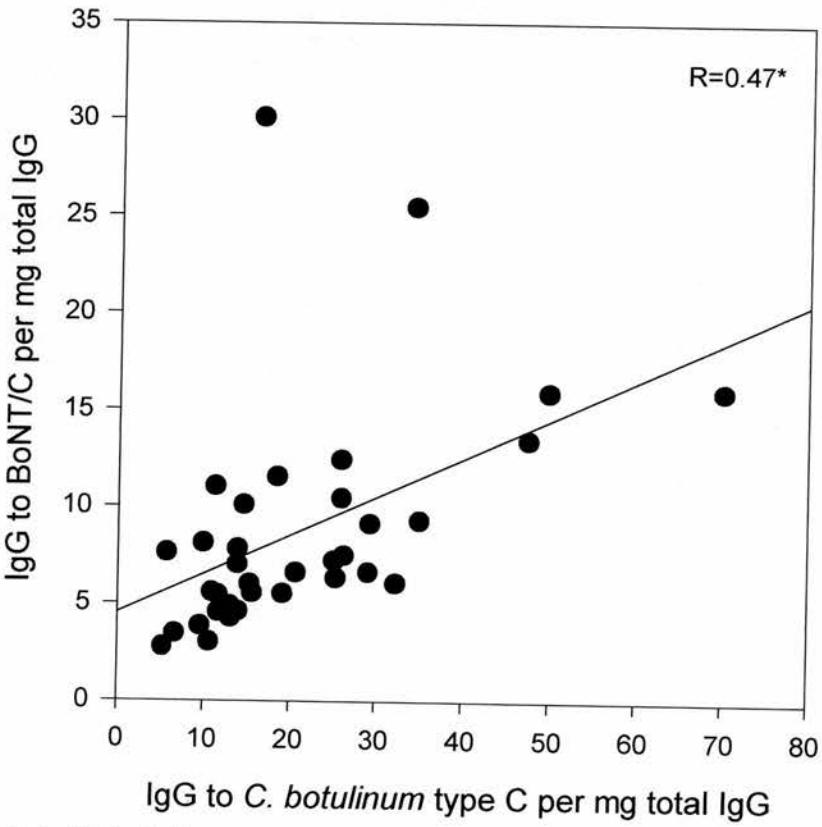


Figure 7.22: IgG to *C. botulinum* type C against IgG to BoNT/C in milk of 36 mares, (a) IgG expressed as OD readings, and (b) expressed as OD/mg total IgG.
*Correlation is significant at the 0.01 level.

Greater correlation was observed between IgA to BoNT/C and surface antigens in milk (R=0.84) (Fig. 7.23) compared to the IgG response (R=0.5) (Fig. 7.22). The correlation was comparable to that observed for the IgA response to BoNT/C and surface antigens observed in the colostrum samples (R=0.84) (Fig. 7.21). There was a higher level of correlation between the IgA response to BoNT/C and surface antigens in the milk of mares in contact with EGS (R=0.89), compared to those that had not been in contact (R=0.73) (Table 7.4).

7.1.6 Correlation between surface antigens of *C. botulinum* type C and *C. novyi* type A

There was a correlation between IgG to the surface antigens of *C. botulinum* type C and IgG to the surface antigens of *C. novyi* type A in the colostrum (R=0.84) (Fig. 7.24). There was also a correlation between the levels of IgA, in colostrum, to surface antigens of *C. botulinum* type C and IgA to surface antigens of *C. novyi* type A (R= 0.78) (Fig. 7.24). However, there was little or no correlation observed between either the IgG or IgA response to the surface antigens in the milk (results not shown).

A higher mean OD, in colostrum and milk samples, was seen to the surface antigens of *C. botulinum* type C, compared to the surface antigens of *C. novyi* type A, in both the ELISAs to detect IgG and IgA to these antigens (Table 7.3).

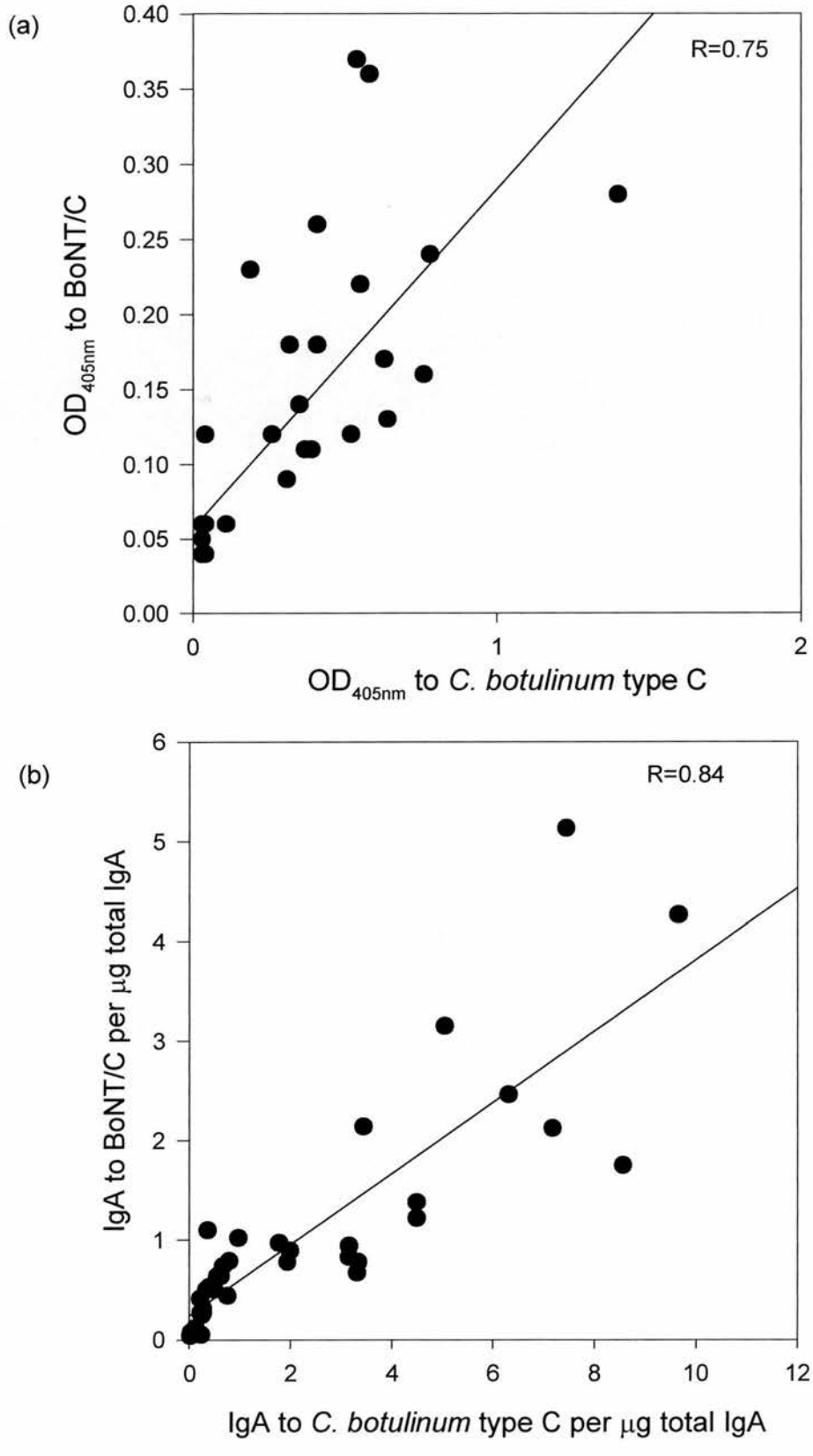


Figure 7.23: IgA to *C. botulinum* type C surface antigens against IgA to BoNT/C in milk of 36 mares. (a) IgA expressed as OD readings, and (b) IgA expressed as OD/μg total IgA. Correlation is significant at the 0.01 level.

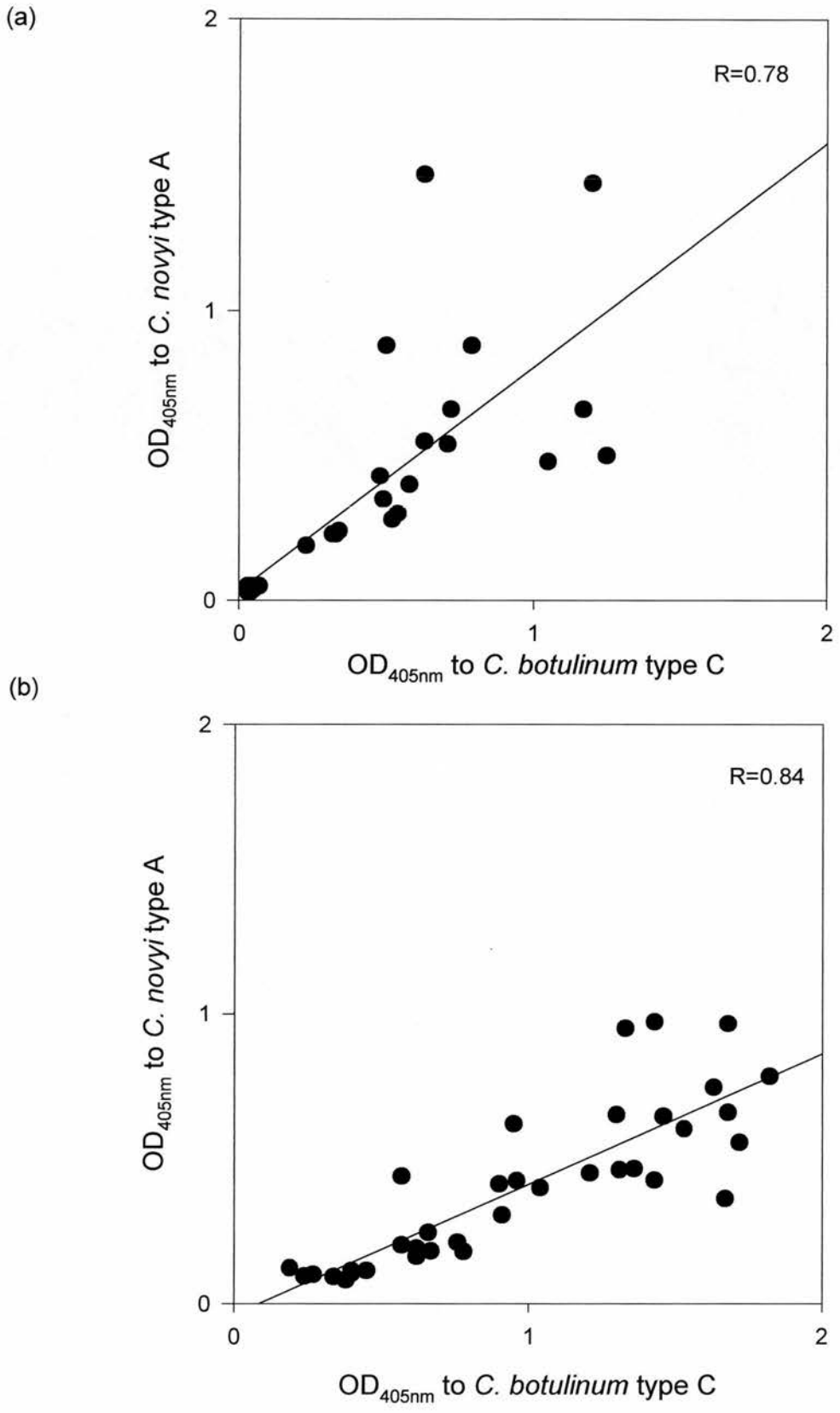


Figure 7.24: (a) IgA to *C. botulinum* type C against IgA to *C. novyi* type A in colostrum, and (b) IgG to *C. botulinum* type C against IgG to *C. novyi* type A in colostrum. Correlation is significant at the 0.01 level.

7.1.7 Longitudinal study of the levels of specific and total antibodies over the course of the suckling period.

Six mares were chosen to investigate the levels of specific IgG and IgA to *C. botulinum* type C surface antigens and BoNT/C, and total IgG and IgA, over the course of the suckling period. Three of the mares had been "in contact" with grass sickness and three had no known previous exposure to cases of grass sickness.

There was a drop in total IgG (Fig. 7.25a) and total IgA concentration (Fig. 7.25b) from the colostrum sample to the first milk sample collected at two weeks post-parturition, in each of the six mares. Milk samples were collected at four weeks and then at four weekly intervals until weaning. There was little variation in total IgG and IgA levels in the milk from two weeks until weaning (Fig. 7.25).

A similar pattern was also seen in the levels of specific antibodies. There was a drop in specific antibody levels between the colostrum sample and the first milk sample, and then little variation in the milk until weaning in all six mares (Fig. 7.26).

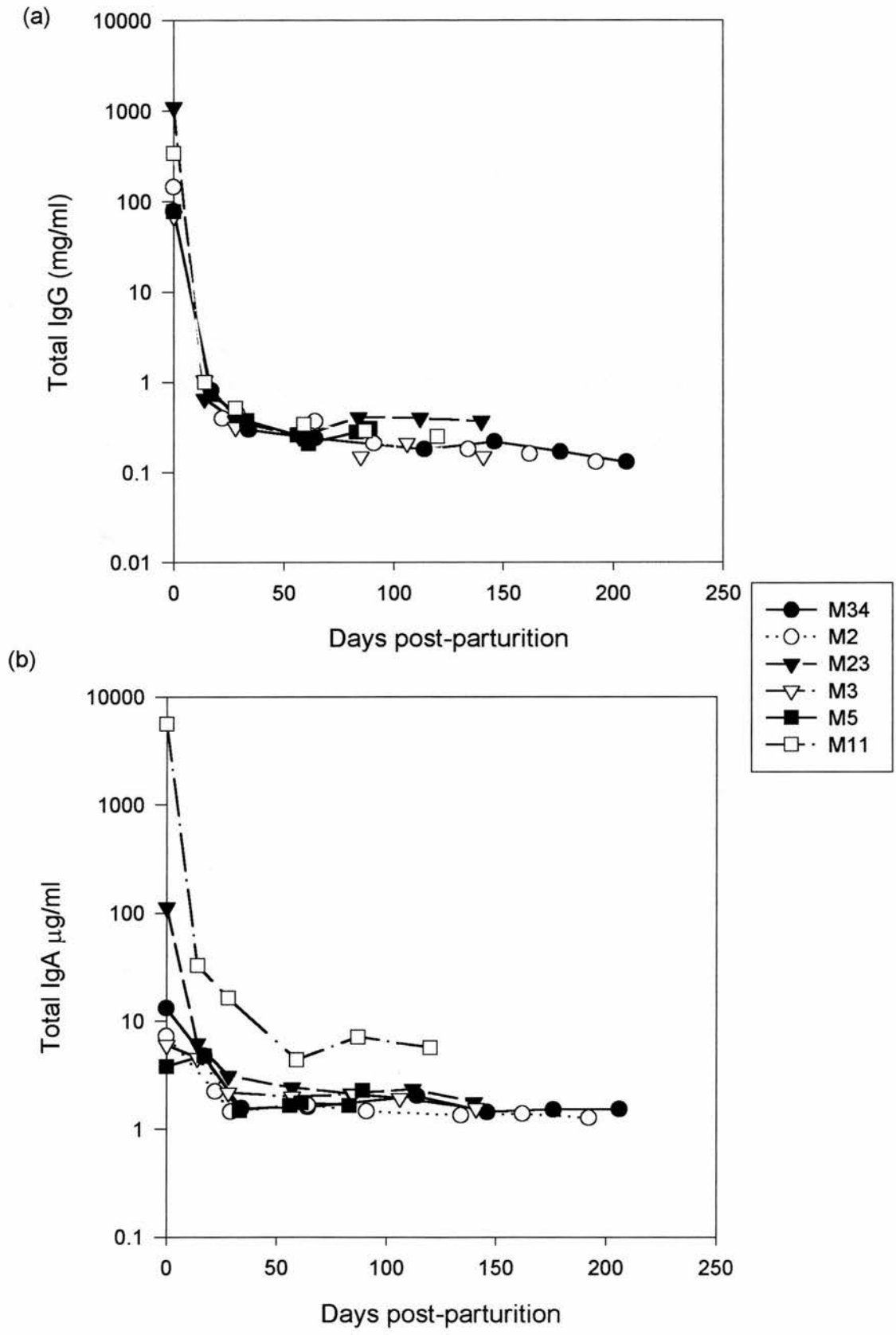


Figure 7.25: (a) Total IgG and (b) total IgA in the colostrum and milk of six mares over the course of the suckling period.

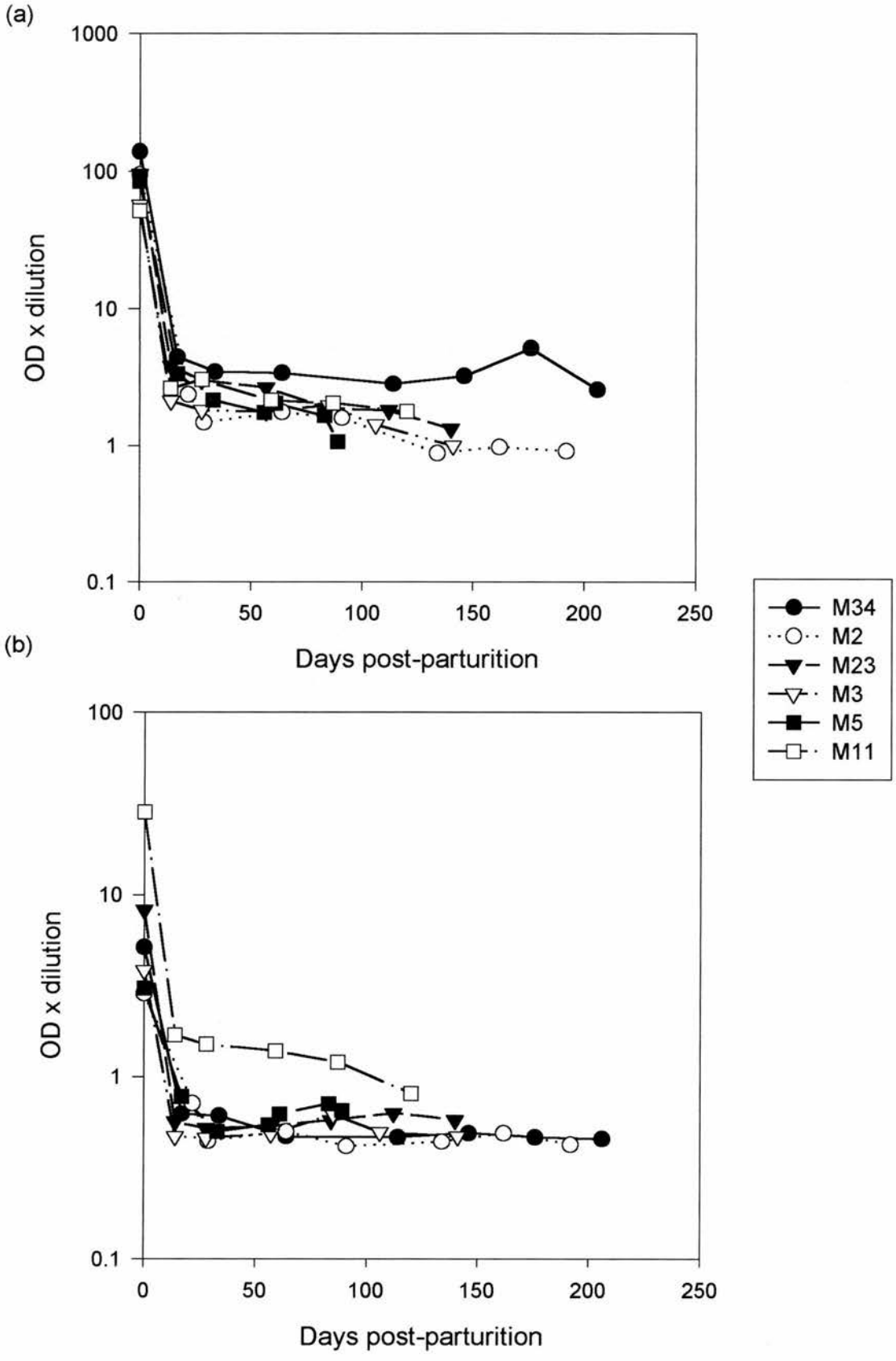
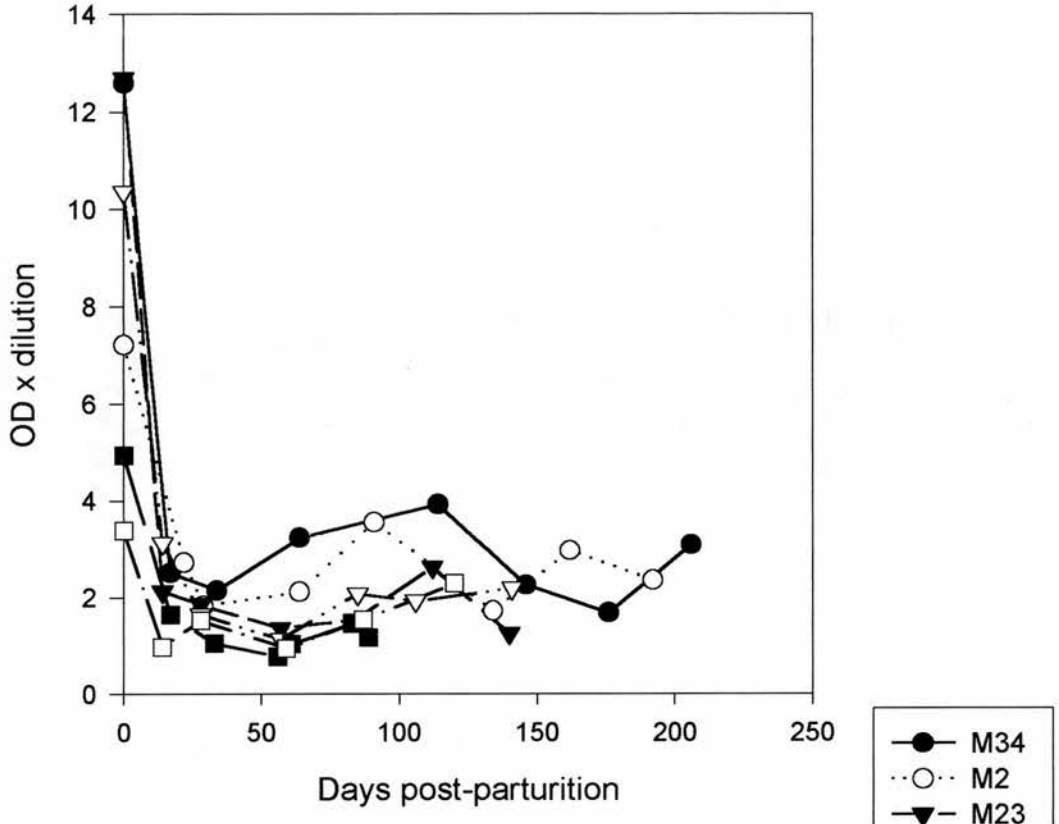
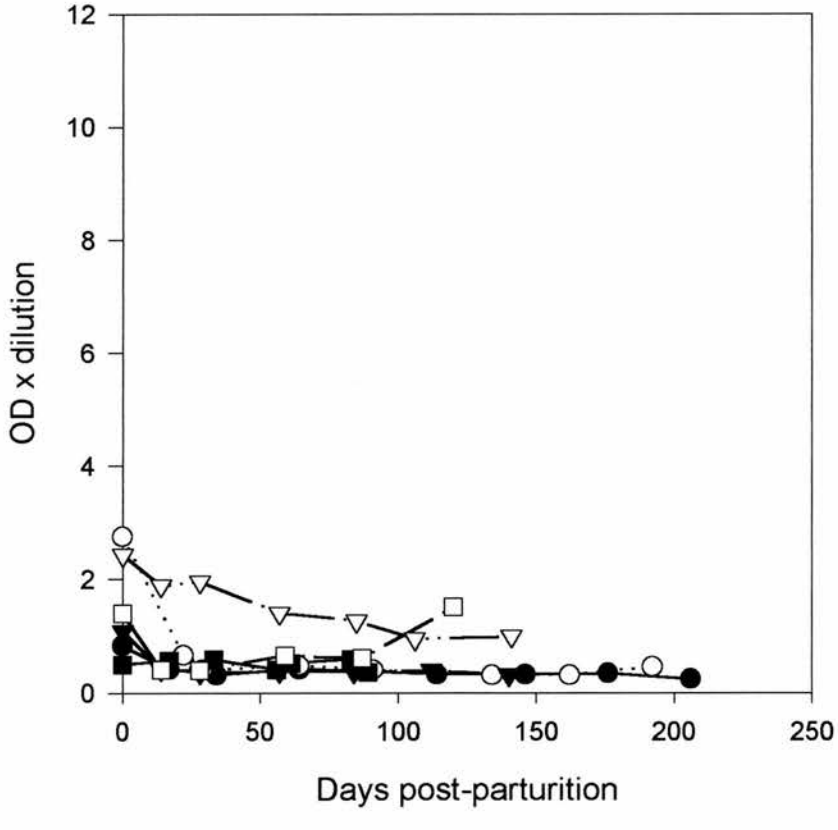


Figure 7.26: (a) IgG to *C. botulinum* type C surface antigens, (b) IgG to BoNT/C, (c) IgA to the surface antigens and (d) IgA to BoNT/C in the colostrum and milk of mares over the course of the suckling period.

(c)


14
12
10
8
6
4
2
0
OD x dilution
0
50
100
150
200
250
Days post-parturition
M34
M2
M23
M3
M5
M11

(d)


12
10
8
6
4
2
0
OD x dilution
0
50
100
150
200
250
Days post-parturition

7.2 Discussion

7.2.1 Development and optimisation of assays

Many problems were encountered in both the development and optimisation of the assays to detect total and specific IgG and IgA in equine colostrum and milk. Limited availability of anti-equine immunoglobulin reagents was a significant problem. The lack of commercially available directly conjugated anti-equine IgA, meant the use of a primary detecting antibody in the ELISA for total IgA. Although an "extra layer" can improve the sensitivity of an ELISA, in this ELISA it raised problems of cross-reactivity and non-specific binding between the reagents, and further limited the choice of suitable reagents. For example, use of a capture antibody and primary detecting antibody of the same host species was not possible due to the conjugate being able to bind to the capture antibody. Using a mouse monoclonal anti-equine IgA antibody as the capture antibody, with a polyclonal rabbit antiserum as the primary detecting antibody, and an anti-rabbit conjugate gave the best results with little or no non-specific binding, either between conjugate and capture antibody or conjugate and sample.

A commercial RID kit was available for determining total IgA. However, due to the expense of the kit it was uneconomical to assay all the samples using the kit, and so this was used to quantify one colostrum sample as a standard.

Fewer problems were encountered with the ELISA for total IgG as there was a directly conjugated anti-equine IgG antiserum available. However, one disadvantage of this ELISA is that it may also detect other isotypes of immunoglobulin, as both the capture antibody and conjugate are raised against the light chain as well as the heavy chain of IgG.

Another problem was the lack of commercially available equine IgG or IgA for use as a positive control in the ELISAs. Therefore these had to be developed from equine serum and colostrum, and quantified before use.

Optimising the ELISAs for specific IgG and IgA to the surface antigens and to BoNT/C was difficult due to the lack of a known positive and negative control. A variety of samples had to be assayed in order to establish the optimum dilution for an assay for screening all samples. In addition, there was a limited availability of BoNT/C coated plates, which restricted the number of samples that could be initially titrated.

7.2.2 Specific antibodies and total immunoglobulins in colostrum and milk

IgG to both *C. botulinum* type C surface antigens and BoNT/C was detected in the colostrum of all 36 mares involved in this study, irrespective of whether the mares had previously been in contact with grass sickness. The presence of these specific antibodies in the colostrum, suggests that maternal antibodies may play a role in the protection of foals from the proposed aetiological agent of grass sickness.

There is no significant transplacental transfer of immunoglobulins in the horse due to the diffuse epitheliochorial nature of the placenta. Passive protection for the foal is therefore all colostrum derived. Colostrum is formed by the selective concentration of serum immunoglobulins in the mammary gland, just prior to parturition (Jeffcott, 1974a). IgG is the major immunoglobulin component in equine colostrum, and is all serum derived. IgA is a minor component of colostrum and is part serum derived

and part locally synthesised in the mammary gland. 58% of mares in this study had IgA to the surface antigens and BoNT/C in their colostrum.

Colostrum is absorbed by the foal, in the first 24 hours after birth, through specialised intestinal epithelial cells. The colostral immunoglobulins enter the circulation and provide systemic passive protection. After birth, there is a rapid turnover of the foal's epithelial cells and by 24-36 hours post-birth the intestinal epithelium is replaced by mature cells, a process named "closure" (Jeffcott, 1975). The foal can no longer absorb macromolecules from the gut, directly into the circulation.

Colostrum derived antibodies, absorbed through the intestinal tract, enter the foal's circulation reaching a peak at about 18 hours (Jeffcott, 1974b). Circulating colostrum-derived antibody then declines, reaching a very low level at about one to two months after birth (McGuire and Crawford, 1973) and becoming undetectable by five months (Jeffcott, 1974b). Levels of colostral derived IgA and IgM decline at a quicker rate reaching very low levels at 16-24 and 6-16 days post-birth respectively (McGuire and Crawford, 1973).

The foal can begin its own immunoglobulin synthesis after about two weeks (Jeffcott, 1974b), reaching protective levels after about a month, coinciding with the decline in colostrum-derived IgG. Genetic immunodeficiencies usually become apparent at around one-two months. Production of IgG by the foal does not approach adult levels until about four months (Jeffcott, 1974b). During this time, the foal receives milk from the mare, which offers additional passive immunological protection from environmental microorganisms, particularly enteric pathogens.

IgA to *C. botulinum* type C surface antigens was detected in the milk of 53% of the mares and 39% of the mares had IgA to BoNT/C in their milk. Seventeen mares had no detectable IgA in the milk to either surface antigens or BoNT/C, and 15 of these did not have detectable IgA in the colostrum to these antigens. IgA is the major immunoglobulin component in milk and is locally produced in the mammary gland (Jeffcott, 1975). Although the immunoglobulins in milk cannot be absorbed into the circulation, they do provide the foal with local passive protection in the GI tract. Therefore, some but not all foals in this study, also received local passive protection as well as systemic protection against *C. botulinum* type C. The longitudinal study suggested that when specific antibodies were detected in the milk, the levels remained more or less constant throughout the suckling period. The foals therefore would continue to receive local passive protection up to weaning.

The presence of locally produced IgA in the milk to *C. botulinum* type C and BoNT/C may be evidence of recent exposure of these mares to the organism and its toxin. There is thought to be a "gut - mammary gland link" with exposure to a micro-organism in the gut resulting in a local immune response in the mammary gland, thus protecting the foal from a specific enteric pathogen (Newby et al, 1982). Whilst the local IgA response in the gut is thought to be relatively short-lived, the specific antibodies produced in the mammary gland persist for longer (Evans et al, 1980).

Local passive protection against *C. botulinum* type C in the GI tract may prevent intestinal colonisation with this organism, whilst enabling the foal to establish its own local immune response against the organism. Foals that do not receive local passive protection against *C. botulinum* type C may be more susceptible to colonisation by this organism. Conversely, they may not be exposed to this organism during the suckling period, and therefore may be more susceptible to

colonisation by this organism and effects of the toxin later in life, due to the absence of an already primed local immune response. It is unlikely that the presence of local passive protection prevents the exposure and development of an active local immune response by the foal. It has been shown that breast-feeding does not inhibit the response to the oral poliovirus vaccine (Deforest et al, 1973 cited in Newby et al, 1982). Finally, there is the possibility that some horses are unable to make a local IgA response to the organism and toxin, making them more susceptible to this disease. Certainly there appears to be a genetic based susceptibility to grass sickness, however the demonstration of IgA to BoNT/C in the gut of some horses with grass sickness (Chapter 6), suggests that the inability to mount a local immune response cannot account for all of this recognised susceptibility.

There were no significant differences in levels of specific IgG or IgA to BoNT/C and *C. botulinum* type C surface antigens in either colostrum or milk between mares that had or had not been in contact with grass sickness. This is in agreement with the serological study of the presence of specific IgG in serum to these antigens (Chapter 5). This investigation showed that although there were statistically significantly higher levels of specific antibodies in horses that were in contact with grass sickness compared to those with the disease, there were no significant differences between horses not known to be in contact with grass sickness and those that had been in contact with the disease. However, the classification of mares in the milk study into two groups should be considered. "In contact", in the milk study was defined as co-grazing land at the time a case of grass sickness occurred, or subsequently grazing the land after a case had occurred. Only seven mares had actually been co-grazing with a case of grass sickness. The majority of "in contact" mares were grazing land where grass sickness had not occurred for

many years. The mares defined as "not in contact" were not known to have grazed land where grass sickness had occurred. As we do not know whether there is a difference in distribution of *C. botulinum* type C between fields where grass sickness has occurred and those where it has not, it may be that the majority of horses are exposed to this organism.

Results for specific antibody levels were expressed as OD levels and also as OD per mg of IgG or μg of IgA in the sample, to take into account variation in amount of immunoglobulin present. Some immunoglobulin might have been lost during transit of samples to the laboratory, thus decreasing the amount of specific antibody available for detection by ELISA; expression of specific antibody as a proportion of total would therefore take this into account. However, variation in total immunoglobulin content will naturally occur between horses, and as the amount of specific antibody present is the important factor when considering protection against a specific pathogen, irrespective of the total immunoglobulin levels present, the results were also expressed as OD readings.

A high level of correlation both between IgG to BoNT/C and IgG to surface antigens and between IgA to BoNT/C and IgA to surface antigens in colostrum demonstrates exposure of the mares to both the organism, reflected by the response to surface antigens, and to the BoNT/C. A high level of correlation between the IgA responses to these antigens was also seen in milk. There was a higher level of correlation between the immune response to these antigens in mares that had been in contact with grass sickness compared with those that had no known contact with the disease. This may indicate a higher frequency of exposure to toxin-producing organisms in the mares that had been exposed to grass sickness. Expression of the

results as a proportion of total IgG or IgA increased the level of correlation, as this corrected for extremes in the distribution points.

Correlation of IgG and IgA levels in the colostrum between the surface antigens *of C. botulinum* type C and *C. novyi* type A validates the use of the surface antigens of the latter organism as a surrogate marker for the former organism, in previous serological assays (Chapter 5). However, little correlation was seen with IgG and IgA in the milk between the surface antigens, and therefore perhaps *C. novyi* type A is a less useful surrogate marker for investigating local immunity to *C. botulinum* type C. Local immunity may be directed at organism-specific antigenic epitopes and not cross-reactive epitopes.

The level of IgG and IgA, in the colostrum and milk, was higher to the surface antigens of *C. botulinum* type C, compared to *C. novyi* type A. The surface antigens of these organisms were extracted by the same method and the ELISA plates were coated with the same concentration, therefore the results should be comparable. The higher antibody levels to *C. botulinum* type C may reflect increased exposure of the mares in the study to this organism.

IgG is the major immunoglobulin component of equine colostrum, quantified as between 9 to 80mg/ml, depending on the investigator (McGuire and Crawford, 1973; Rouse and Ingram, 1970; and Pahud and Mach, 1972). The mean value determined in this study for total IgG content of colostrum was 116.2mg/ml, which is slightly higher than previously determined values. In this study, an ELISA was used to determine the concentration of IgG in colostrum, whereas previous studies used single radial immunodiffusion assays. The use of different techniques may account for the differences in antibody levels between the studies.

IgA is a relatively minor component of colostrum making up only 10% of the total immunoglobulin content (Pahud and Mach, 1972). The level of IgA in colostrum is reported to be between 1.5mg/ml and 9mg/ml depending on the investigator (McGuire and Crawford, 1973 and Pahud and Mach, 1972), again detected by single radial immunodiffusion assay. The ELISA detected a mean concentration of 1.40mg/ml for total IgA in colostrum in our study, comparable with previously reported findings.

The immunoglobulin content of colostrum falls dramatically in the first 12 hours post-parturition, particularly after suckling by the foal. Rouse and Ingram (1970) reported a drop in total IgG from 45mg/ml measured at 0-3 hours post-parturition to 7.26mg/ml at four to eight hours post-parturition. Similarly McGuire and Crawford (1973) showed a decrease in colostral IgG from 19.2mg/ml to 2.7mg/ml, one day after birth. These findings not only demonstrate the importance of early suckling by the foal (usually two to three hours after birth) to ensure adequate passive protection, but also may account for the differences for immunoglobulin content reported in the literature. As colostrum is secreted only once, the immunoglobulin content of colostrum may also be decreased by premature lactation prior to birth (Jeffcott, 1974b).

IgA is the major immunoglobulin component of milk, making up 67% of the total immunoglobulin, compared to 10% of the total immunoglobulin in colostrum (Pahud and Mach, 1972). Again, there is variation in the reported concentrations of IgA and IgG in milk. The concentration of IgA in milk is reported in the literature as 0.8mg/ml or 0.85mg/ml and that of IgG as 0.35mg/ml or 0.64mg/ml (Pahud and Mach, 1972 and McGuire and Crawford, 1973). Our study found a mean concentration of

0.02mg/ml total IgA, and 1.95mg/ml total IgG in the 36 milk samples. The IgA concentration was lower than previously reported findings, whereas the IgG concentration was higher. This may reflect the differences in techniques for determining the concentration values. The levels of IgG detected by the ELISA may be higher than the actual amount of IgG present, as both the coating antibody and the conjugate were polyclonal anti-equine IgG (H+L). The fact that these antisera could also bind to equine light chains may result in cross-reaction with other immunoglobulin isotypes.

In summary the differences in IgG and IgA concentrations found in colostrum and milk compared to published findings may be due to the difference in technique (ELISA compared to RID), different times of sampling colostrum (up to 12 hours post birth) and also the many different breeds of horse used. It has been reported that different breeds produce different concentrations of immunoglobulins in the colostrum and serum (Rouse and Ingram, 1970). In this study, milk was collected from a wide variety of breeds of horses and no attempt was made to separate the mares on the basis of breed.

There is little published information on specific antibody titres after the colostral period. Browning et al (1991), investigated antibody to rotavirus in the serum and milk of normal, infected and vaccinated horses. They found that the specific antibody levels in the milk did not differ significantly over the period of seven to 70 days postpartum and inferred that a rise during this period would indicate a response to an infection or vaccination. Findings in our study also showed little change in the specific antibody levels to the surface antigens or the toxin in the milk of the six mares investigated. This may be evidence of non-infection over the suckling period. However, the specific antibody levels did not drop significantly in

these samples and so alternatively this may indicate constant or repeated exposure to these specific antigens in order to maintain a specific immune response. Antibody levels in milk persist for longer than the equivalent immune response in the gut. Where small variation was observed in specific antibody levels it was not possible to conclude whether it was a significant increase, i.e. resulting from exposure.

In summary, the demonstration of specific IgG to surface antigens and to BoNT/C in the colostrum of all the mares investigated is evidence that foals may be protected systemically from the proposed aetiological agent of equine grass sickness. Demonstration of IgA to these antigens, in the milk of some of the mares, is evidence that some foals also receive local passive protection against the organism and the toxin. However, in the absence of challenge experiments, it is not known whether the levels of specific maternal antibodies detected in the colostrum and milk are present at a protective level.

Chapter Eight

Conclusions

This thesis has investigated the hypothesis that equine grass sickness is caused by toxicoinfection with *C. botulinum* type C. As an initial investigation, this study has taken a broad approach in order to obtain evidence for the involvement of *C. botulinum* type C in equine grass sickness. Thus, detection of BoNT/C, isolation and characterisation of BoNT/C-producing organisms from the GI tract and detection of systemic and mucosal antibodies, in horses with and without grass sickness, have been studied.

In the absence of a known aetiological agent, epidemiological studies suggested that grass sickness might have an infectious aetiology, albeit an unconventional one, whilst the pathology and experimental findings suggested a toxic aetiology. Toxicoinfection with *C. botulinum* type C could potentially fulfil the criteria of both an infectious and toxic aetiology.

Detection of BoNT/C and BoNT/C-producing organisms in the GI tract of horses with grass sickness, has demonstrated a strong association between *C. botulinum* type C and the disease. While these results support the involvement of a toxicoinfection with *C. botulinum* type C with the disease, they cannot demonstrate that this is the cause of grass sickness. However, the detection of IgA to BoNT/C in the GI tract of horses with grass sickness, not only confirms recent exposure to the toxin, but also suggests exposure to the toxin, at least in horses with acute grass sickness, prior to the onset of GI stasis.

The clinical, pathological and epidemiological similarities between grass sickness and dysautonomias, more recently recognised in other animals, such as cats, hares and rabbits, suggested a common aetiological agent. During the course of this thesis, BoNT/C and BoNT/C-producing organisms have also been detected in the GI tract of cats, hares and a rabbit, all with dysautonomia. This provides evidence of common exposure to *C. botulinum* type C and BoNT/C in animals with dysautonomia, thus both supporting a role for a common aetiological agent in these diseases and supporting the hypothesis that the common aetiology in these dysautonomias is toxicoinfection with *C. botulinum* type C. However, it should be emphasised that the investigation of animals, other than horses, with dysautonomia, was a very minor part of this thesis. Therefore the role of C. botulinum type C in these dysautonomias requires further investigation, with more control animals, to confirm these initial findings.

Epidemiological evidence suggests that an immune response to the aetiological agent occurs in grass sickness. Investigation of the immune response of horses to *C. botulinum* type C and BoNT/C demonstrated that horses that had been in contact with grass sickness had higher serum IgG levels to BoNT/C and surface antigens, than horses that had the disease. This suggests that horses in contact with grass sickness have been subclinically exposed to *C. botulinum* type C and BoNT/C and have made a systemic immune response. Epidemiological findings have demonstrated that prior contact with a case of grass sickness is associated with a ten-fold decrease in likelihood of the disease (Wood et al, 1998). The results of the investigation of systemic antibody levels, suggest that this decrease in likelihood of disease might be due to the presence of protective systemic antibodies. In addition, grass sickness is rarely seen in foals prior to weaning. The presence of antibodies

to *C. botulinum* type C and BoNT/C in all colostrum samples investigated, suggests that foals are passively protected from *C. botulinum* type C and BoNT/C.

In theory, if grass sickness is caused by toxicoinfection with *C. botulinum* type C, then it should be preventable by vaccination against both the organism and its toxins. However, whilst the detection of serum and colostrum antibodies to *C. botulinum* type C and BoNT/C, suggest their role in preventing the disease in horses that have been in contact with grass sickness and suckling foals, respectively, there is no evidence that the levels of antibodies detected in these studies are protective. Challenge experiments would be necessary to establish this. The demonstration of a significantly higher level of mucosal IgA to BoNT/C in the GI contents of horses with grass sickness, compared to controls, raises the issue of whether a specific mucosal immune response to this toxin can be effective in the prevention of and recovery from the disease. As the enteric nervous system is severely damaged in grass sickness, it would appear preferable to have a primed mucosal immune response to limit the exposure of enteric neurons to the toxin. Further investigation of the mucosal immune response is therefore required to establish whether a potential vaccine should also stimulate mucosal immunity.

Further work is required to ascertain the involvement of other toxins potentially produced by *C. botulinum* type C, such as C2, C3 and the *C. novyi* alpha toxin, in this disease. In addition, the source of infection with *C. botulinum* type C should be established. At present, the prevalence of Group III organisms in the GI tract of the equine population is not known. Systemic antibody levels in horses to the surface antigens of Group III organisms suggest common exposure. However, Group III organisms, all identified as *C. novyi* type A, were only isolated from animals that had been exposed in some way to dysautonomia. The future use of PCR might prove to

be a more sensitive technique for determining the carriage of Group III organisms in the equine GI tract. Investigation of pasture for the presence of Group III organisms and the relevant phages, should demonstrate whether there is an environmental reservoir. Identification of the presence of toxin genes in organisms in the GI tract and in the environment should provide evidence of whether toxigenic or non-toxigenic organisms are carried. Equine grass sickness may result only when a phage, carrying a toxin gene, is introduced into a microorganism that is normally non-toxigenic.

The alteration of the GI environment, by an unknown factor, is considered important for the establishment of toxicoinfection with *C. botulinum* type C, and hence the onset of disease. However, investigation of this trigger factor was beyond the remit of this thesis. Without determining this predisposing factor, there may be difficulties in reproduction of the disease experimentally, and hence difficulties in establishing toxicoinfection with *C. botulinum* type C as the cause of the equine grass sickness. Research is currently being carried out in areas which may lead to the identification of a trigger factor, for example, antioxidant levels in herbage (McGorum et al, 2000), and cyanide levels in clover on pastures (B. McGorum, personal communication). The study of other animals with dysautonomias likely to be caused by the same aetiological agent as grass sickness should enable the establishment of an experimental model for the disease, in which to investigate the hypothesis. However, differences in trigger factors between the dysautonomias in other animals might well occur. Establishment of in vitro cultures of equine enteric neurons should enable the investigation of the toxic effects of the implicated toxins – BoNT/C, C2 or *C. novyi* alpha toxin –on these neurons.

In conclusion, the findings of these initial investigations support the hypothesis that toxicoinfection with a Group III botulinum organism, producing BoNT/C, is involved in the aetiology of equine grass sickness. Additionally, a basis has been established for further research into the role of *C. botulinum* type C in the pathogenesis of this disease.

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The association of *Clostridium botulinum* type C with equine grass sickness: a toxicoinfection?

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Keywords: horse; *Clostridium botulinum* type C; neurotoxin; equine grass sickness; dysautonomia; C2 toxin; C3 exoenzyme

Summary

The cause of grass sickness, an equine dysautonomia, is unknown. The disease usually results in death. Gastrointestinal (GI) dysfunction is a common clinical manifestation in all forms of the disease. It is generally thought that equine grass sickness (EGS) is caused by an ingested or enterically produced neurotoxin which is absorbed through the GI tract. *Clostridium botulinum* was first implicated as a causative agent when it was isolated from the GI tract of a horse with EGS in 1919. The aim of the present study was to investigate the hypothesis that EGS results from toxicoinfection with *C. botulinum* type C: growth of the bacterium in the GI tract with production of toxin (BoNT/C). Ileum contents and faeces from horses with EGS were investigated for BoNT/C, and indirectly for the presence of *C. botulinum* type C, and compared with control samples from horses without EGS. BoNT/C was detected directly by ELISA in the ileum of 45% (13/29) of horses with EGS compared to 4% (1/28) of controls, and in the faeces of 44% (20/45) of horses with EGS compared to 4% (3/77) of controls. Levels of up to 10 µg toxin/g wet weight of gut contents were observed. The one control horse with detectable toxin in the ileum had been clinically diagnosed as having acute EGS, but this was not confirmed by histopathology. The organism was detected indirectly by assaying for BoNT/C by ELISA after enrichment in culture medium. *C. botulinum* type C was shown to be present in 48% (14/29) of ileum samples and 44% (20/45) of faecal samples from horses with EGS, compared with 7% (2/27) of ileum samples and 8% (6/72) of faecal samples from controls. These results support the hypothesis that EGS results from a *C. botulinum* type C toxicoinfection.

Introduction

Equine grass sickness (EGS) is a frequently fatal dysautonomia of unknown aetiology. The disease is most commonly reported in Great Britain, particularly Scotland, but EGS also occurs in other parts of Northern Europe, Australia and the Falkland Islands (Cottrell *et al.* 1999). Mal seco, an equine dysautonomia, which is probably identical to EGS, is seen in Argentina and Chile (Uzal and Robles 1993).

The clinical, pathological and epidemiological features of the disease are well documented (Gilmour 1987; Edwards 1987; Doxey *et al.* 1991a,b; Wood *et al.* 1998). There is severe neuronal degeneration and widespread neuronal loss in both the enteric and autonomic nervous systems. EGS can present in 3 forms classified on the basis of duration and severity of clinical signs. The acute and subacute form invariably result in death or euthanasia; some selected chronic cases have recovered with appropriate treatment, the mainstay of which is thorough nursing care (Doxey *et al.* 1998). The most common clinical signs are dysphagia and gastrointestinal stasis/dysfunction. Horses with the subacute and chronic forms rapidly lose weight and condition.

The cause of grass sickness is still unknown. Many putative causative agents have been considered since the disease was first recognised early this century, including fungal, plant and chemical toxins (Greig 1942; Doxey *et al.* 1990). Tocher *et al.* (1923) presented the first evidence to support a hypothesis that *Clostridium botulinum* was the cause of grass sickness. A bacterium morphologically and toxigenically resembling *Bacillus botulinus* (now *Clostridium botulinum*) was first isolated from the gastrointestinal (GI) tract of a case of EGS in 1919 and, subsequently, from the spleens of a number of horses with grass sickness (Tocher *et al.* 1923). However, further investigation and a subsequent vaccine trial failed to convince Tocher's contemporaries that this was the causative agent, despite some reduction in the incidence of the disease in vaccinated animals. Horses were vaccinated with a combination of toxin and antitoxin (Tocher *et al.* 1923), which had been prepared from known strains, and not from the organisms isolated from the EGS cases. The antitoxin was in fact from a US human source.

It is proposed that the disease is caused by an ingested or enterically produced neurotoxin that is absorbed through the GI tract, severely damaging the enteric nerves. There is a correlation between the clinical severity and the extent and distribution of enteric neuronal damage (Doxey *et al.* 1992; Pogson *et al.* 1992; Scholes *et al.* 1993) with the greatest neuronal loss usually observed in the ileum. It is thought that the neurotoxin reaches the peripheral autonomic ganglia via the circulation and/or by retrograde axonal transport (Griffiths *et al.* 1994).

There are several factors that point to the disease being of an infectious aetiology, with the development of an immune response to the causative organism. EGS is seasonal with cases predominantly seen between April and July and as the name suggests it is associated with grazing. Older horses and those that have spent longer on a particular pasture have a decreased

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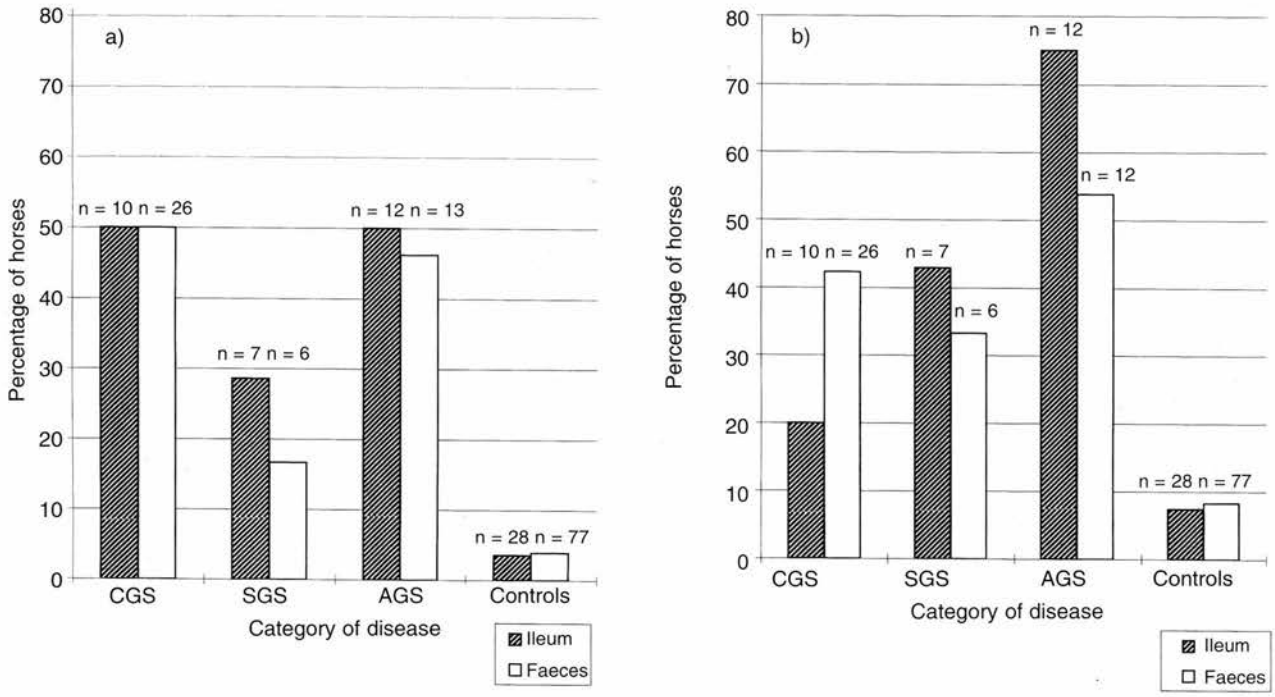


CGS = chronic grass sickness; SGS = subacute grass sickness; AGS = acute grass sickness

Fig 1: Percentage of horses with detectable BoNT/C in the ileum and faeces (a) directly, i.e. before enrichment, and (b) after enrichment. The number on top of each bar represents the number of horses in each category on which calculation of the percentages has been based.

incidence of grass sickness. Prior contact with a case of EGS is associated with a 10-fold reduction in the likelihood of the disease (Wood *et al.* 1998).

In 1994, J. K. Miller (personal communication) hypothesised that the aetiopathogenesis of EGS results from a toxicoinfection by an organism of the *Clostridium botulinum* group III phenotype (serotype C or D). The organism grows in the GI tract with elaboration of toxin *in vivo*, a situation similar to human infant botulism. *C. botulinum* type C predominantly affects animals and can produce 3 toxins: C1, (BoNT/C), C2 and C3.

BoNT/C is a botulinum neurotoxin that inhibits the release of acetylcholine at cholinergic nerve terminals. It prevents neurotransmitter release by specific proteolysis of syntaxin (Schiavo *et al.* 1995) and SNAP-25 (Foran *et al.* 1996; Osen-Sand *et al.* 1996; Williamson *et al.* 1996). Syntaxin and SNAP-25 are both synaptosomal proteins that are involved in synaptic vesicle exocytosis. These proteins are conserved within neuronal synapses, and, therefore as would be expected, botulinum neurotoxins can inhibit other neurotransmitters besides acetylcholine *in vitro* (Bigalike and Habermann 1981; Mackenzie *et al.* 1982; Foran *et al.* 1996; Williamson and Neale 1998). BoNT/C is severely neurotoxic to mouse spinal cord neurones (Williamson and Neale 1998) and rat hippocampal and cortical neurones (Osen-Sand *et al.* 1996) in culture. This neurotoxicity is unique to BoNT/C; the other clostridial neurotoxins block neurotransmission, but only BoNT/C can cause overt neuronal degeneration (Williamson *et al.* 1995). It is therefore possible that BoNT/C can cause damage to a wide range of neurones *in vivo*.

C. botulinum type C differs from the other botulinum serotypes in that it can produce a further 2 toxins, both of which have ADP-ribosylating activity. C2 is a binary toxin which ADP-ribosylates the G-actin form of β/γ cytoplasmic and γ smooth muscle actin (Mauss *et al.* 1990), resulting in the depolymerisation of the actin microfilament network. C3 is an exoenzyme which ADP-ribosylates the Rho-family of low molecular weight GTP binding proteins required for the organisation of the microfilament network (Hara-Yokoyama *et al.* 1994). C2 and C3, by their effect on the cytoskeleton of the cell, can bring about ultrastructural disarray. C3 can cause neuronal degeneration *in vitro* (Williamson and Neale 1998).

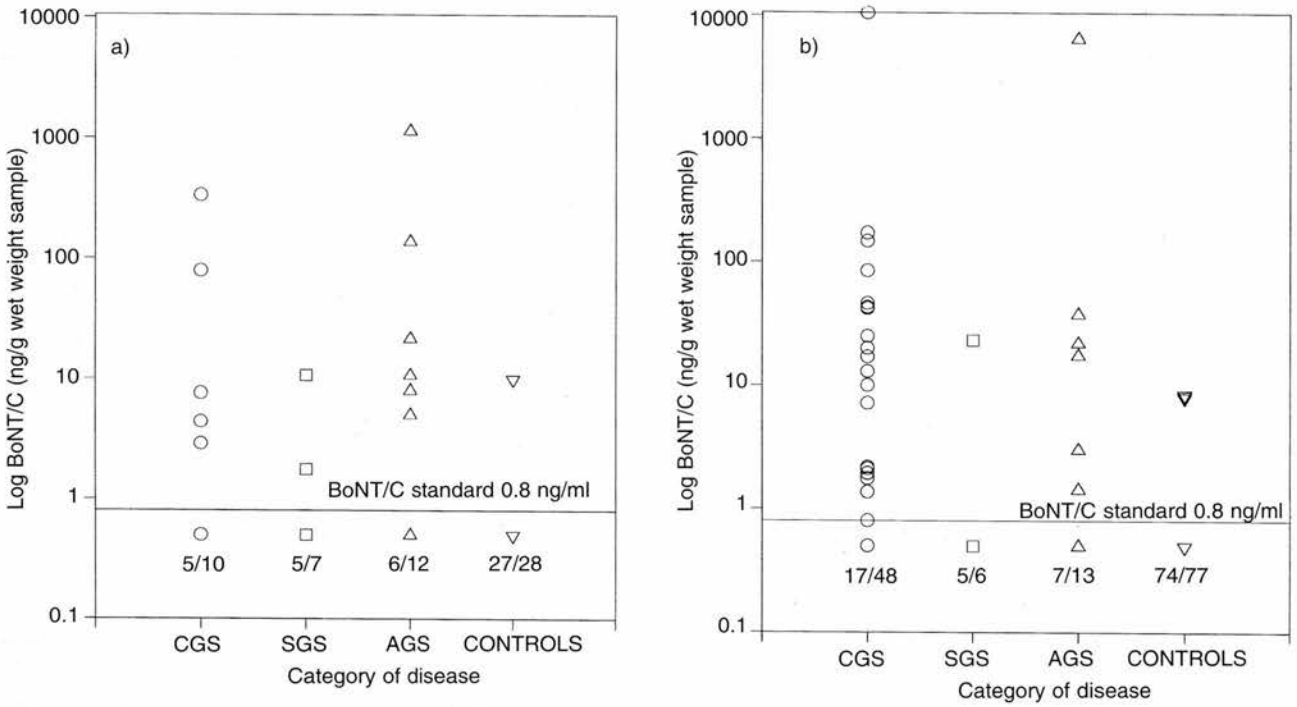
A pilot serological study (J.K. Miller *et al.* unpublished data) suggested that a serum IgG response was made to surface antigens of *C. novyi type A* (used as a surrogate marker, as it is phenotypically identical to *C. botulinum* type C/D [Poxton 1984]) and to BoNT/C during chronic grass sickness.

The aim of the present study was to investigate intestinal contents and faeces from horses with EGS for the presence of BoNT/C. These were compared with control samples from horses without EGS, to determine whether there is evidence for a role for *C. botulinum* type C in the aetiology of this disease. Preliminary findings of this study were presented at the 10th Biennial Symposium of the Society for Anaerobic Microbiology in Cambridge in July 1997, and results published in the Proceedings (Poxton *et al.* 1997).

Materials and methods

Clinical ileal and faecal samples

Intestinal contents were taken from the distal ileum at *postmortem* and rectal faecal samples were taken *antemortem*. Ileal samples were taken from 30 horses with EGS (acute, n = 12; subacute, n = 8; chronic, n = 10) and 28 controls (normal, i.e. apparently healthy horses, n = 2; a horse which had EGS in the past but not at the time of sampling, n = 1; clinical nonEGS cases with gut dysfunction, colic, n = 1; and without gut



CGS = chronic grass sickness; SGS = subacute grass sickness; AGS = acute grass sickness

Fig 2: Scatterplot showing the range of BoNT/C concentrations directly detected in (a) ileum samples and (b) faecal samples. There is a statistically significant difference in the levels of toxin detected in the ileum of the CGS group and controls (P = 0.001), between the SGS group and controls (P<0.05) and between the AGS group and controls (P<0.001). There is a statistically significant difference in the levels of toxin directly detected in the faeces between the CGS group and controls (P<0.001), and between the AGS group and controls (P<0.001). The number of samples in each category which did not have detectable toxin are represented as a proportion of total sample number in each group, by the numbers below the reference line of the BoNT/C standard of 0.8 ng/ml, on the graph. The detectable limit of the assay is considered to be equivalent to 0.8 ng/ml of the BoNT/C on the standard curve from the ELISA.

dysfunction, n = 15; and with unknown diagnoses, n = 9).

Faecal samples were taken from 45 horses with EGS (acute, n = 13; subacute, n = 6; and chronic, n = 26) and 77 controls (normal, i.e. apparently healthy horses, n = 24; horses which had EGS in the past but not at the time of sampling, n = 3; healthy horses in contact with clinical EGS cases, n = 34; clinical nonEGS cases with gut dysfunction, colic, n = 4, ileus, n = 1, and without gut dysfunction, n = 11).

Processing of samples for toxin detection

1) Direct detection: weighed specimens (1–10 g) were infused into a known volume (5 or 10 ml, depending on consistency of sample) of phosphate-buffered saline, pH 7.2, with 0.2% gelatin (PBSG), and held overnight at 4°C. After vortexing, the liquid portion was removed and centrifuged at 3,800 ***g*** for 20 min. The supernate was subsequently collected and stored at -20°C.

2) Enrichment: approximately 1–3 g of specimen was added to 15 ml prereduced CMC-1 medium (developed by Centre for Applied Microbiology Research (CAMR)[1] and incubated at 30°C for 5 days. The samples were centrifuged at 3,800 ***g*** for 20 min and the supernatant was collected and stored at -20°C.

Enzyme-linked immunosorbent assay (ELISA): a sandwich ELISA for the detection of *C. botulinum* type C1 (BoNT/C) had been developed by CAMR[1]. A polyvalent guinea pig antiserum raised (supplied by CAMR) against the purified neurotoxin was used as a capture. The micro plates (Nunc F8 Polysorp)[2] were coated (100 µl/well) overnight at 4°C with 5–10 µg/ml of the antiserum diluted in phosphate buffered saline, pH 7.4 (PBS). Plates were washed 3 times with PBS containing 0.1% Tween 20 (PBS-T). Plates were postcoated with PBS-T containing 5% foetal bovine serum (PBS-TF) for a minimum of 1 h at 37°C and then washed 3 times in PBS-T. Sample supernates were assayed in duplicate at 3 dilutions (undilute, 1 in 2, and 1 in 4 for direct toxin detection; 1 in 5, 1 in 25 and 1 in 125 for enriched samples) in PBS-TF (100 µl/well). Purified BoNT/C diluted in PBS-TF to 100 ng/ml, 20 ng/ml, 4 ng/ml and 0.8 ng/ml was included as a standard to enable comparison between plates and quantification of results. The plates were incubated at 37°C for 90 min and then washed 3 times with PBS-T. The anti-BoNT/C guinea pig IgG (supplied by CAMR)[1], conjugated to HRP, was used at a dilution of 1 in 300 in PBS-TF (100 µl/well). Plates were incubated for 90 min at 37°C. After washing 3 times in PBS-T, the substrate (3, 3', 5, 5'-tetramethyl-benzidine dihydrochloride tablets[3] dissolved in phosphate-citrate buffer pH 5.0 with 2 µl 30% H_2O_2/10 ml was added (100 µl/well). The reaction was allowed to develop at room temperature for 30 min or until the background became detectable. The reaction was stopped by the addition of 50 µl/well 2 mol/l H_2SO_4. Plates were read in an Anthos plate reader at 450 nm, with a reference at 620 nm.

Samples were considered positive if the mean optical density was equal to or greater than that for the 0.8 ng/ml BoNT/C standard. A standard curve was used to quantify the toxin concentrations in samples that had been infused in PBSG,

TABLE 1: Detection and quantitation of BoNT/C, before and after enrichment, in multiple faecal samples taken from horses with CGS

Horse reference number	Duration of disease (days)	Day sampled	BoNT/C detected before (ng/g wet weight sample)	BoNT/C after enrichment	Outcome
97/150	75	5	No	No	Euthanasia
		75	7.2	Yes	
98/540	47	38	No	No	Recovered
		47	42	Yes	
97/545	40	6	No	Yes	Recovered
		39	1.36	No	
97/623	67	23	147.5	No	Recovered
		66	43	Yes	
97/834	54	1	No	No	Recovered
		22	No	No	
		39	0.7	No	
		43	1.75	No	
97/987	47	10	No	No	Recovered
		14	2.1	No	
		21	171	Yes	
		28	25	Yes	
		35	17	No	
		41	10	No	

correcting for the original dilution factors. The amounts of BoNT/C in samples, which had been enriched in CMC-1, were not quantified in this way due to amplification of toxin in the enrichment process.

Statistical analysis

The levels of BoNT/C detected directly in the ileal and faecal samples were compared statistically between the categories of EGS disease and the controls by the Mann-Whitney U test.

Results

Direct detection of BoNT/C in the ileum and faeces

Overall BoNT/C was directly detected in 45% (13/29 horses) of ileum contents from EGS cases compared with 4% (1/28 horses) of controls and in 44% (20/45 horses) of EGS faecal samples compared with 4% (3/77 horses) of controls.

BoNT/C was detected in the ileum contents of a greater proportion of chronic (50%) and acute cases (50%) than subacute cases (29%). It was detected in the faeces of a greater proportion of chronic (50%) and acute cases (46%) than subacute cases (17%). This is summarised in Figure 1a.

The one control case with detectable toxin in the ileum had been initially diagnosed clinically as acute EGS. However, histopathology at *postmortem* failed to show the characteristic lesions, and the final diagnosis was uncertain. The 3 control horses, which had detectable toxin in their faecal samples, included 2 orthopaedic cases, one of which had had chronic EGS 16 months previously, and a horse with surgical colic.

Quantitation of BoNT/C in the ileum and faeces

The amount of BoNT/C detected in the ileum and faeces was quantified as ng of BoNT/C/g wet weight sample. Comparisons between samples should be made with caution as the concentration of toxin is calculated using the wet weight of the sample and there will be some variation in consistency between samples. The volume of gut contents can vary significantly between horses, which may also affect toxin detectability.

There was a statistically significant difference in the levels of toxin detected in the ileum, between the CGS group and controls ($P = 0.001$), between the SGS group and controls ($P<0.05$) and between the AGS group and controls ($P<0.001$). There was a statistically significant difference in the levels of toxin directly detected in the faeces between the CGS group and controls ($P = 0.001$) and between the AGS group and controls ($P<0.001$).

The highest level of BoNT/C (10430 ng/g) was detected in the faeces of a horse with chronic grass sickness of 15 days duration, which was sampled at *postmortem*. The ileum of this horse also contained detectable toxin (79.2 ng/g). The one control horse in which BoNT/C was detected in the ileum had a low level of toxin (10 ng/g wet weight sample), but note that this horse had clinical signs typical of EGS.

Longitudinal study of horses with CGS

Twelve chronic cases had more than one faecal sample collected. Eleven of these horses were initially negative for BoNT/C and 5 of these had detectable levels in later faecal samples (the others remained negative). The twelfth horse had detectable BoNT/C in both faecal samples. Table 1 shows how the levels of detectable toxin in the faeces of the 6 horses varied over the course of the disease. Five of the 6 horses recovered; the sixth was subjected to euthanasia, and a level of 335 ng/g BoNT/C was detected in the ileum at *postmortem*. Of the 6 horses that had no detectable toxin, 3 recovered and 3 were subjected to euthanasia (one of these had toxin detectable after enrichment).

Detection of BoNT/C after enrichment of samples in culture medium

Detection of BoNT/C after enrichment of samples in culture medium indicates that *C. botulinum* type C was present in a sample. After enrichment in CMC-1 medium, BoNT/C was

detected in 48% (14/29) of ileum samples and 44% (20/45) of faecal samples of EGS cases, and 7% (2/27) of ileum samples and 8% (6/72) of faecal samples from controls (Fig 1).

The 2 control horses, out of 27, with detectable BoNT/C in the ileum after enrichment were the horses clinically diagnosed as AGS but not histopathologically confirmed, and the other a horse with a hepatic disorder. The 6 horses, of 72 controls, with detectable BoNT/C in the faeces after enrichment were 2 orthopaedic cases (one of which had had grass sickness 16 months previously), 2 healthy horses, a horse in contact with an EGS case, and a horse with ileus.

Ileal samples from AGS cases had a higher proportion of positive samples after enrichment (75%) than SGS (43%) and CGS (20%). Faecal samples from AGS cases also had a higher proportion of positive samples after enrichment (54%) than CGS (42%) and SGS (33%). The relationships between detectable BoNT/C in the ileum and faeces both before and after enrichment between the different groups of horses are illustrated in Figure 1b.

Not all samples that had detectable levels of toxin directly were positive for BoNT/C after enrichment, and *vice versa*. Table 1 shows which of the consecutive faecal samples from the chronic EGS cases were positive after enrichment.

Detection of BoNT/C in ileum and/or faeces by direct detection and/or enrichment.

When the detection methods are combined for individual horses i.e. when toxin can be detected in the ileum and/or faeces by direct detection and/or enrichment, then 74% of acute cases, 67% of subacute cases and 67% of chronic cases have detectable toxin compared to 10% of controls.

Discussion

The results show that there is a strong association between BoNT/C toxin in the equine gastrointestinal tract and a clinical diagnosis of EGS. Toxin was detected *antemortem* in faecal samples from chronic cases and *postmortem* in ileum and faecal samples of all categories of EGS. Toxin was not detected in all the samples from EGS cases. However, this may be a reflection on the sensitivity of the assay; the ELISA is approximately 100 times less sensitive than the conventional mouse (lethality) bioassay that can be used for toxin testing. Horses are extremely sensitive to botulinum toxins, and even the mouse bioassay cannot detect toxin in the serum of horses with classical botulism (Johnston and Whitlock 1987). Only a very small amount of toxin may be needed to exert a clinical effect. The absence of detectable toxin in the ileum and faeces may also be due to the rapid absorption of BoNT/C from the gut, destruction by proteases or neutralisation of the toxin by mucosal antibodies.

Another factor that may affect the ability to detect BoNT/C is the point in the disease at which the sample was taken (as well as the volume of gut contents present at time of sampling). This is of particular relevance with respect to the chronic cases, where the disease can last from several weeks to several months. The sampling of chronic horses over the course of the disease showed that the toxin could be detected in the faeces of 6 horses towards the end of the disease, whereas 5 of these horses had been initially negative when tested earlier. Five of these 6 horses were considered to have recovered but still had toxin in their faeces close to or on the date that they were sent back to their owners. The ability to detect toxin in the faeces of these horses is therefore not necessarily an indication for a poor prognosis. Horses may be able to tolerate a subclinical level of toxin in the faeces: toxin produced in the large intestine may be poorly absorbed, or destroyed, in healthy horses. Colonisation of infant mice with *C. botulinum* does not cause illness despite the detection of up to 2000 mature mouse intraperitoneal 50% lethal doses of toxin in the colon and caecum. This is thought to be due to localisation of the toxin to the colon where it would be poorly absorbed (Sugiyama 1980).

The presence of toxin in the ileal is probably a more significant finding with respect to clinical outcome. BoNT/C was detected, pre-enrichment, in the ileal contents of only one of 28 controls. As noted previously, this horse had been clinically diagnosed as an acute EGS case; however, the characteristic neuronal lesions of EGS were not found on histopathological investigation at *postmortem*. This case highlights the need to investigate the possible role of *C. botulinum* type C in cases presenting with clinical signs similar to EGS. This sample, as well as an ileal sample from a horse with a hepatic disorder, had toxin present after enrichment, demonstrating the presence of *C. botulinum* type C in the upper GI tract.

It is interesting to note that the acute EGS cases had a higher mean level of toxin in the ileum and faeces, than the other categories. It is possible that acute EGS may result from exposure to a large amount of toxin, whereas the chronic form may result from the exposure to smaller amounts of toxin over a longer period of time (Doxey *et al.* 1995). The clinical severity and duration of the disease correlates to the extent of enteric neuronal damage. Enteric neuropathy is widespread and more severe in acute cases but in chronic cases the neuronal loss is less and tends to be localised to the distal small intestine (Scholes *et al.* 1993). BoNT/C was detected in both the ileum and faeces of approximately 50% of acute EGS horses. This suggests that either there are toxin-producing organisms along the length of the gut, or toxin has been produced in the ileum and has passed along the length of the gut prior to the formation of the impactions that often occur.

We are investigating the hypothesis that EGS is a toxicoinfection with *C. botulinum* type C. The diagnosis of a toxicoinfection requires the demonstration of the presence of both toxin and toxin-producing organism in the affected animal, as is the case for infant botulism (Hatheway 1979). The presence of *C. botulinum* type C in the samples is demonstrated by detecting the toxin in enrichment culture supernates. The production of this neurotoxin is encoded on a prophage that can be lost or acquired during growth and sporulation. Therefore the detection of BoNT/C after enrichment does not necessarily mean that an organism was producing toxin *in vivo*, and conversely an organism that was producing toxin *in vivo* may not be detected in culture due to loss of the prophage. *C. botulinum* type C is a particularly fastidious organism, and successful culture and isolation can be very difficult. A significant number of samples do demonstrate the presence *C. botulinum* type C, for example, 75% of ileal samples from acute cases, which would support the hypothesis of a toxicoinfection. The samples, which do not have detectable toxin after enrichment, could be due to the absence of organism in the enriched sample, prophage instability, or inhibition of growth or toxin production by other organisms present.

Only 4% of 72 control faecal samples contained an organism capable of producing BoNT/C after enrichment. This may indicate the low carriage of toxigenic organisms in the normal equine population, or it may suggest that there are factors preventing detection of the organisms. We are developing a

PCR technique for the more accurate assessment of the carriage of this organism in the equine population.

There are some differences in opinion as to the clinical relevance of *Clostridium botulinum* isolated from the GI tract of animals (Kinde *et al.* 1991). The organism is found commonly in soil samples and aquatic sediments (Hatheway 1990) but its distribution can vary considerably even locally (Smith 1975). Different types of *C. botulinum* are found in different geographical areas; for example, *C. botulinum* type B is predominant in the soils in the eastern US, and consequently this serotype is the major cause of adult equine botulism and shaker foal syndrome in that area (Johnson and Whitlock 1987). Type C is a cause of equine botulism in Europe, but is less common in the US despite being the predominant type isolated from soils in Florida (Johnston and Whitlock 1987). Types C and D have been described as obligate parasites of animals and birds, seldom being isolated from the soil unless from an area where birds or domestic animals congregate (Smith 1975). Spores of *C. botulinum* are commonly found in the faeces of herbivores without clinical signs (Kinde *et al.* 1991). However, it has been reported that spores are rarely isolated from normal, healthy horses that are not at risk from botulism, and that toxin has not been isolated from the faeces of a mature animal *antemortem* (Johnston and Whitlock 1987). Here, we have demonstrated that both the organism and the toxin can be detected in the faeces of horses *antemortem,* as well as the ileum of horses *postmortem*; these animals did not present with classic botulism, but equine grass sickness.

It is important to address the question why these horses do not present with classic signs of botulism. EGS does share some clinical similarities to botulism, particularly in the acute form (Tocher *et al.* 1923; Greig 1928). However, many of the neuromuscular signs characteristic of classical botulism are not observed. The chronic and subacute forms develop a very tucked up 'greyhound-like' abdomen (Gilmour 1988). This is strikingly similar to the 'wasp-waist' seen when laboratory mice are inoculated with botulinum toxins.

Equine Grass Sickness is thought to be associated with intestinal colonisation of *C. botulinum* with *in vivo* production of toxin(s), rather than ingestion of preformed toxin, which is the cause of classic botulism. Consequently the enteric nervous system of the horse is exposed to a high dose of locally produced toxin, causing severe neuronal damage and initiating the GI dysfunction associated with the disease. We propose that there is a concentration gradient effect away from the focus of toxin production, perhaps generated by axonal transport, with the more peripheral ganglia being affected to varying degrees. If this is the case, then it is possible that insufficient neurotoxin reaches the peripheral cholinergic synapses at the neuromuscular junction to cause the neuromuscular signs observed in botulism (in classic botulism the preformed toxin is ingested and absorbed rapidly, becoming systemic). Hodson *et al.* (1984) observed that changes in the coeliacomesenteric ganglion resemble those of classic retrograde degeneration after axotomy. This supports the hypothesis that the primary lesion is in the GI tract. The analogous toxicoinfectious botulism in humans (infant botulism, and the extremely rare adult form) has a different clinical presentation from that caused by ingestion of preformed toxin. Disruption or immaturity of normal flora, disturbance in gut motility, and compromisation of the gastric acid barrier, can all predispose to intestinal infection with *C. botulinum* in man (Chia *et al.* 1986). Botulism has also been observed in man where there is mainly cholinergic autonomic dysfunction with no neuromuscular involvement (Jenzer *et al.* 1975).

The clinical signs of EGS may result from the involvement of the 3 toxins produced by *C. botulinum* type C. To date we have only assayed for C1 (BoNT/C); we do not have assays for the C2 and C3 toxins at present. However, the C2 and C3 toxins, together with the *in vivo* growth of the organism, may be responsible for the difference in clinical symptoms between EGS and botulism. C2 toxin has been shown to cause hypotension, haemorrhage, cytotoxicity and increase in vascular permeability (Aktories *et al.* 1986). In the intestine it has been shown to cause intestinal secretion, fluid accumulation, histopathological changes (Ohishi and Odagiri 1984) and inhibition of contraction of guinea pig ileum myenteric plexus longitudinal muscle preparation (Mauss *et al.* 1989). However, its role in the pathogenesis of disease *in vivo* has yet to be determined.

Ultrastructural investigation of the neuronal lesions of EGS has indicated marked abnormalities of the neuronal cytoskeletal, cytoplasmic and secretory proteins with disruption or loss of the Golgi-associated membranes (Griffiths *et al.* 1993). It is possible that the C2 and C3 toxins could cause the ultrastructural damage observed. Actin-polymerisation has a role in governing secretion from neural cells and consequently is thought to play some role in neurotransmission (Viviani *et al.* 1996). All 3 of the toxins produced by *C. botulinum* type C could therefore affect neurotransmission in some way, either by their effect on the cytoskeleton or the secretory vesicles of the exocytic pathway. The neurotoxicity of BoNT/C (Williamson and Neale 1998) may account for some of the neuronal degeneration that is observed in EGS.

The enteric cholinergic neurones from the ileum of horses with acute EGS exhibit altered cholinergic mechanisms, with a reduction in the release of acetylcholine (Murray *et al.* 1994). Botulinum neurotoxins inhibit the release of acetylcholine from cholinergic nerves. The prokinetic drug Cisapride, an indirect cholinergic agent, facilitates the release of acetylcholine from the postganglionic nerves of the myenteric plexus in the gut. It has been shown to be of some therapeutic benefit in the treatment of selected cases of chronic EGS (Milne *et al.* 1996). Acetylcholine release is stimulated from the remaining morphologically normal neurones and increases gut motility.

This is, to some extent, a reinvestigation of the hypothesis originally put forward by Tocher *et al.* in 1923. Their work was not widely accepted at the time. One of the major criticisms was their inability to prove conclusively that the organism they had isolated from grass sickness horses was *Bacillus botulinus*; they could only say that it morphologically and toxigenically resembled this organism. However, serotype C of *C. botulinum* was first identified only in 1922 (Bengston 1922; Seddon 1922). This was almost certainly not the serotype which was vaccinated against in 1922, particularly as the antitoxin used was of US human origin. *C. botulinum* type C had not been identified as a cause of human botulism at that point, and there have only been 5 suspected human type C cases since (Sonnabend *et al.* 1985). The neurotoxins of different types of *C. botulinum* are not immunologically cross-reactive.

At this point, it is not possible to say whether *C. botulinum* type C is the primary cause of EGS, a causal cofactor, or whether the organism grows and produces its toxins as a secondary event due to reduced gut motility. Five faecal samples (one horse with ileus and 4 with colic) and 2 ileal samples (one horse with colic

and one with an unknown diagnosis) from horses with GI dysfunction, but not grass sickness, have been assayed to date. This has shown the presence of toxin in one faecal sample from a horse with colic and, in one ileal sample, from a horse which had presented clinically with AGS, but the diagnosis was unknown after *postmortem*.

In summary, we propose that EGS is a toxicoinfection by *C. botulinum* type C with toxin production and absorption predominantly in the ileum. The organism is either normally present in the large intestine, and overgrows into the ileum, or the organism is taken up in spore form and is able to germinate in the ileum, following a change in the environment of the GI tract due to an environmental trigger. Horses are notoriously sensitive to changes in diet, and grass sickness is associated with grazing and a change in pasture. The trigger therefore could be a nutritional one, affecting the gut flora, and facilitating the colonisation of the intestine with *C. botulinum* and its subsequent toxin production.

The disease severity is likely to be influenced by an interaction of factors such as the amount of toxin the horse is exposed to, the local mucosal and systemic immune response to the toxin, the immune status at the onset of disease and the inherent susceptibility of the horse. Whether *C. botulinum* type C is the cause of grass sickness or whether it is involved as a secondary event, the detection of high levels of toxin in the ileum at *postmortem*, and *antemortem* in faeces in horses with EGS, together with the known sensitivity of horses to botulinum toxins, supports a significant role for the involvement of *C. botulinum* type C in equine grass sickness.

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VETERINARY MICROBIOLOGY (ORAL PRESENTATION)

Is Equine Grass Sickness (Mal Seco?) a Form of Botulism?

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Key Words: Horse, dysautonomia, *Clostridium botulinum*

Introduction

Equine grass sickness (EGS) is an often fatal dysautonomia of unknown aetiology. It is found in Northern Europe, especially in the eastern half of the United Kingdom. It is characterized by gastrointestinal dysfunction due to degeneration of the enteric autonomic nerves, particularly in the ileum. EGS presents in acute, subacute and chronic forms [1]. The disease mal seco, reported in horses from the Patagonia region of Argentina and Chile, appears clinically and pathologically equivalent to EGS [2].

EGS was first recognized in Scotland in 1907, but despite a great deal of research the aetiology of the disease remains a mystery. The epidemiology suggests that EGS may be caused by a toxin, probably of microbial origin, which is either ingested or produced in the intestine following a nutritional trigger. Clustering of cases occurs and the disease is seasonal. The disease generally occurs in younger animals, and animals imported from a non-endemic area to an endemic area appear more susceptible to EGS — both of which suggest the development of immunity. The disease is restricted to certain geographical areas, even down to certain pastures on the same property.

The link between botulism and EGS was made as early as the 1920s when Tocher *et al.* [3] claimed that EGS was caused by *Clostridium botulinum* (possibly type A). This claim was discounted when vaccine field trials, with vaccine imported from the U.S.A., failed to show complete protection. However, vaccinees had partial protection [4]. In the 1970s unpublished anecdotal evidence was suppressed that suggested that *C. botulinum* type C was involved. In

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the early 1990s, Jean Robb, after researching the old literature, suggested to us that a reappraisal of the role of *C. botulinum* should be made. Later, J.K. Miller formulated a hypothesis (unpublished) that EGS is a toxico-infection — based on the likely consequences of the action of toxin(s) of C/D *C. botulinum* on the enteric autonomic nervous system. There were several parallels to infant botulism in humans (e.g. dysphagia, constipation, diminished appetite, spectrum of severity).

Clostridium botulinum type C produces three exotoxins: (1) C1 toxin (BoNT/C, the classical neurotoxin) is a protease that cleaves syntaxin when inserted into the lipid bilayer of a presynaptic membrane, inhibiting release of neurotransmitters. Its action in classical botulism is flaccid paralysis. However, its likely action when produced locally, and possibly slowly, in the GI tract is unknown. (2) The C2 toxin, a binary toxin, ADP-ribosylates the G-actin of beta/gamma cytoplasmic and gamma smooth muscle actin, resulting in depolymerization of actin microfilament network. (3) The C3 toxin ADP-ribosylates the rho family of low molecular weight GTP-binding proteins which are required for the organization of the microfilament network. All three toxins could affect neurotransmission by their effects on the cytoskeleton or the secretory vesicles of the exocytic pathway, but could these toxins cause the clinical and pathological changes of EGS?

The aim of the current study was to investigate whether BoNT/C could be detected in clinical cases of EGS and compare this with control animals to see if there was any correlation between presence of toxin and symptoms of EGS. In a parallel serological study, antibodies were assayed to BoNT/C, and to cell surface antigens of *Clostridium novyi*, a non-botulinum-toxin-producing species identical in cell surface composition to group III *C. botulinum* [5]. The results of the serological study are being published elsewhere [6] and preliminary results for both toxin and serology have been published recently [7].

Materials and Methods

Horses investigated

To date 36 cases of clinically confirmed EGS and 45 controls (including contacts) with and without intestinal symptoms have been investigated. If animals have gone to *post mortem*, the contents of the distal ileum and rectum have been collected. Those chronic cases that have survived, as well as many of the controls, have only had faecal samples taken (per rectum).

Immunoassay for BoNT/C

Weighed specimens (1–10g) were infused into a known volume (5 or 10 mL depending on consistency) of PBS pH 7.2 containing 0.2% gelatin, and held overnight at 4°C. After thorough mixing, the heavy debris was allowed to settle, and the liquid phase clarified by centrifugation ($4000 \times g$, 20 min). The supernate was stored briefly at −20°C. A sandwich ELISA developed at CAMR, Porton Down, U.K., and used under licence, was used to measure BoNT/C. Briefly, microwell plates coated with polyclonal guinea pig anti-serum raised against purified BoNT/C were used to detect toxin in the supernates. A standard curve was included on each plate using purified BoNT/C. Captured toxin was detected with guinea pig IgG conjugated to horseradish peroxidase and HRP substrate (3,3′,5,5′, -tetramethyl-benzidine dihydrochloride and hydrogen peroxide). Results were expressed as ng of toxin per g wet weight of sample.

Antibodies to BoNT/C (antitoxin) were assayed in a similar manner, with plates coated at CAMR with purified BoNT/C. The antitoxin in horse serum was detected with anti-equine alkaline phosphatase conjugate.

Results

Detection of C1 toxin (BoNT/C)

Clinical cases and controls have been investigated for the presence of *C. botulinum* type C1 neurotoxin (BoNT/C) in *post-mortem* specimens of contents of distal ileum, and in rectal samples of living horses (Figure 1).

C1 toxin has been detected in the ileum contents of 43% chronic (three of seven), 83% acute (five of six), 50% subacute (two of four) and 7% controls (one of 14), and in the faeces in 65% chronic (11 of 17), 20% acute (two of seven), 20% subacute (one of five) and 3% controls (one of 36). This information is summarized

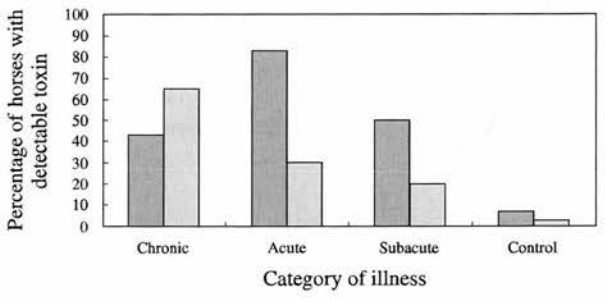

Figure 1. Percentage of horses with detectable BoNT/C in (■) ileum and faecal (□) samples.

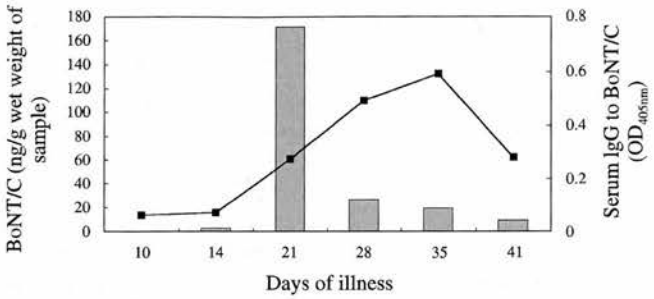

Figure 2. BoNT/C in faeces (□) and serum IgG (–■–) consecutive samples from a case of chronic EGS.

in Figure 1. C1 toxin was detected more often in the ileum contents of more animals with acute or subacute disease than with chronic cases, and, conversely, in the faeces of more of the chronic cases. The single horse where toxin was detected in the ileum contents was admitted with a provisional clinical diagnosis of acute grass sickness. However, the characteristic pathology of EGS could not be observed on necropsy. The single control animal in which toxin was detected in faeces was diagnosed as having colic, and the toxin levels were low.

In parallel serological studies, we have seen rising titres of specific anti-BoNT/C IgG during the course of the illness in chronic cases (an example is shown in Figure 2), but overall, serological data have proved difficult to interpret when single samples have been investigated [5]. The results shown in Figure 2 show the appearance of toxin in faeces followed by apparent seroconversion.

Discussion

We have demonstrated an association between clinical symptoms of grass sickness and BoNT/C in the ileum, but we are aware that we have not yet proved that there is a causal relationship. Although the association is not absolute, the ELISA system that we are employing is not as sensitive as the biological action of botulinum toxin; several samples may have had levels of C1 toxin below the detectable limits.

Currently we are examining the hypothesis that the development of grass sickness involves a nutritional trigger and susceptibility to disease may be determined by the natural defences of the horse, such as the ileal mucosal immunity. It is possible that most if not all horses are exposed to *C. botulinum* type C, but that any toxin produced in the intestine is usually neutralized by mucosal antibodies or destroyed by natural proteases. Grass sickness may develop after intestinal bacteria produce amounts of toxin that cannot be controlled by the normal defences, perhaps subsequent to increased bacterial multiplication. The nutritional trigger is unknown but presumably involves the biochemical status of the grass. Similarly the mechanisms of enteric nerve regeneration remain unclear. In summary, we have assembled both toxicological and serological correlations with EGS that suggest several new avenues worth pursuing.

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